

Natural History Museum rumpus

The British Natural History Museum has taken too short and too jaundiced a view of its own future as a research institution. It should mend its ways, and quickly.

WHAT emerges most clearly from the long controversy about the future of the British Natural History Museum (NHM) is that the management's 'corporate plan', published in February, was a serious error of judgement. Faced with the prospect of a nasty financial squeeze — a shrinking budget and the high cost of maintaining the building in which it is housed — the museum plumped for the wrong solution, that of cutting back on an already inadequate intramural research programme. This is not the first time in the past ten years that British research institutions have been forced by financial pressure to respond in such a way. But usually there has been no choice. At the NHM, on the other hand, it would have been possible to cut back instead on the museum's second function of mounting attractive public exhibitions. The museum seems to have plumped for what it calls its "front-of-house" activities.

If the correspondence appearing in *Nature* is anything to judge by (see, for example, page 406), that reaching the museum directly has probably been even more blistering. It is all very well for Sir Walter Bodmer, the new chairman of the trustees, to imply that the volume of comment excited by the corporate plan is a measure of the regard in which NHM is held (*Nature* 345, 569; 1990), but what is to be said when most of the comment is critical of the decision? What the rumpus has shown is that the rest of the world regards the NHM's collection as a unique resource, which it fears may now be lost to scholarship.

It is only fair to NHM to acknowledge that the fear may prove groundless. If the plan to separate curatorship from research succeeds as the museum intends, the collections may be no less accessible. The much more serious worry is that the reduction of the volume of research activity (by about a fifth) will further attenuate the museum's in-house expertise, compromising its capacity to act as a cheer-leader for taxonomy in Britain or anywhere. To say this does not imply that everything that used to happen at NHM was first-rate, but merely that relinquishing research activities is, in the present British climate, a recipe for permanent abandonment.

Worse still is the superficiality of the language of the corporate plan. The truncated research programme, which it is said will be "responsive to audience needs", has been renamed under headings such as Environmental Quality and Human Health. (In the approving digest of the corporate plan in last week's survey of British spend-

ing on research, "health" is at one place wishfully misprinted as "wealth".) The justification of the latter, according to the new director, Dr Neil Chalmers, is the museum's acknowledged expertise in the identification of the vectors of human diseases (*Nature* 345, 198; 1990). But that is an unconvincing explanation for such a comprehensive title, however trendy. The "front-of-house" seems to have altogether too much influence on what little will in future happen back-stairs.

It is not as if the "front-of-house" will save the museum from the troubles that lie ahead. The temptations in this direction are of course natural; since the imposition of entrance charges, NHM has been collecting more than £2 million a year from its visitors. The hope is that spending more funds on better exhibitions will increase this source of revenue. But there is no chance that the extra income will pay for the cost of earning it. Yet the scale on which funds are to be spent on better exhibitions will not create a temple for the celebration of discovery to match, say, the new Musée de la Cité in Paris. Naturally, there is a chance that better exhibitions will win round some young people to careers in science. But does NHM know enough about its audience, and their susceptibility to exhibitions, to make that likelihood substantial?

What should happen now? There is no chance that the museum will change its mind. Its corporate plan is, in any case, already part of the British government's planning for the future. But even that unfortunate document acknowledges that the real crunch lies two or three years ahead. However buoyant the museum's bookshop and restaurant sales, the forecasts point to a danger that the scale of NHM's activity will be further under pressure two or three years from now. Should not the museum be making the case for relief from that further squeeze right now and — for the sake of its constituents — in public? It should also do more than has yet been done to show that there is substance in its hope that support for research by British academics at the museum will indeed be provided by the research councils, themselves not flush with funds. And if it is true, as museum officials have been saying, that the corporate plan was never intended to persuade the scientific community but only the Office of Arts and Libraries and, ultimately, the British Treasury, should it not now provide a more intelligent statement of its aspirations for the continuation of research at the museum? If the outcome should be a plan that cannot be financed out



of the present budget, it would not be improper that the trustees should set out to find the funds from elsewhere. But this time, the museum should consult its constituency in advance, and not simply produce a document like a rabbit from a hat.

What NHM should have learned from the commotion of the past several weeks is that it is not just another British research institution, as disposable as Kleenex, but one whose research function is genuinely international. The museum should have been particularly stung by those who have donated their personal collections to the museum in the belief that it was a perpetual institution. NHM's collections and their investigation have a better claim on such a status than most other research collections. The trustees would now seem to have a stark choice: either to exert themselves so as to redress the balance between exhibition and research, if necessary making nuisances of themselves in the process, or to resolve to dispose of the collection to some institution elsewhere that will be able to use it properly. □

Computers for sale

The sale of ICL to a Japanese company is not necessarily a bad thing.

INTERNATIONAL Computers Limited (ICL), the British computer manufacturer sold this week to Fujitsu, has a chequered but absorbing history. It is the inheritor of the venture embarked on by the British company Ferranti in the late 1940s which led, by 1949, to the realization of an admired and then technically advanced digital computer. But the expectation that the machine would be the foundation of a successful British computer industry were frustrated by the peculiarly British expectation that ambitious technical developments can be sustained on a financial shoestring. Over the past four decades, the company has had several narrow escapes from catastrophe, but in the past decade has won itself an enviable reputation as an independent manufacturer of mainframe machines equipped with sophisticated software. The partnership with Fujitsu should at least keep the tradition alive.

So why should the rest of Europe be up in arms about the intended merger? The simplistic argument is that it is somehow disloyal to the concept of European unity that a strategically important manufacturer based in Europe should be controlled financially from outside. But that is a little European's view. Is it not far better that Europe should have a financially strong computer manufacturer, able to operate internationally, than that it should be saddled with an implicit obligation to protect ICL (and other European manufacturers) from outside competition? The planned merger, in short, should help to dissuade the new Europe from chauvinistic protectionism. The US Congress, about to embark on another such bout of chauvinism, would be well advised to follow suit. □

Garbage, garbage . . .

The US General Accounting Office, skilled at uncovering waste in public spending, has been beaten by garbage.

"WHAT has four wheels and fries?" quip the wags at the US General Accounting Office (GAO). "A multipurpose truck cross-hauling nonhazardous municipal solid waste and pre-prepared potato foodstuffs which, if a refrigerated unit, is in contradiction of the regulations issued by the Secretary of Transportation as instructed by the House of Representatives bill HR 3386" intone their diligent colleagues, who have spent the past nine months compiling a document that must surely rank among the most inconclusive government reports of all time.

Asked by the investigations and oversight subcommittee of the Committee on Public Works and Transportation to investigate the possibility that the practice of food/garbage cross-hauling — using the same vehicle to transport both components of the human equation — is a risk to health, the GAO has concluded that, "[w]hile federal health and food safety experts contend that the risk of food contamination from cross-hauling with garbage is relatively low . . . [w]e, along with the federal regulators and health experts, believe that current information is not adequate to rule out health risks in transporting food in these trucks". In short, the GAO report is as noncommittal as its health experts.

Each of the document's 44 pages offers a new twist in what is obviously a cunning pastiche of a scientific report. The cover sets the tone: what better title for a groundbreaking report on possible health risks than *Truck Transport: Little Is Known About Hauling Garbage and Food in the Same Vehicles*? Similarly, chapter three — the report's thundering denouement — is simply entitled "Experts Do Not Know Potential Food Contamination Risks From Cross-Hauling Garbage and Food".

But it is all too easy to underestimate the task facing the compilers of the GAO report. At the mysterious request of state officials, two of the four landfill sites at the focus of the survey were out-of-bounds to the researchers. Yet, rather than cravenly finding two other suitable landfills instead, the intrepid accountants persevered, scribbling down the licence numbers of trucks using the off-limits sites. Although they were unable to speak to either the drivers or the operators, the GAO was ultimately successful in taking a photograph of a signpost in the road outside one of these sites, an achievement described triumphantly as "Figure 2.1: Sign Outside Ohio Landfill".

Another classic example of the visual aid is Figure 3.1, which does its best to justify the report's tenuous initial claim that "transporting food in a truck that previously hauled garbage inspires high emotions in many individuals". The accompanying photograph shows a truck full of garbage, allowing the document to reach its only conclusion: "As figure 3.1 depicts, a truckload of loose garbage is a disgusting sight". □

Safety in numbers for Earth Observing System

- Industry report offers redesign
- NASA decries unwanted advice

Washington

OFFICIALS at the National Aeronautics and Space Administration (NASA) last week attacked a draft report that suggests the space agency may be pursuing a poor design for its planned \$40,000 million series of climate monitoring satellites, collectively known as the Earth Observing System (EOS).

The report, by three researchers from the space and technology group of California-based TRW Inc., argues that it would be better to construct EOS as orbiting groups of many small, relatively inexpensive satellites, rather than as a few large, multi-purpose platforms, each the size, complexity and cost of the Hubble Space Telescope.

The recent demoralizing failure of the Hubble's optics, along with suggestions that the proposed space station Freedom will require an impossible number of man-hours for routine maintenance, has added weight to the criticism that large, complex systems invite disaster. But Ray Roberts, EOS programme director, said that the unsolicited advice from TRW, which at present has no major contracts for EOS, reflected "vested interests". He warned against "opportunists" and "NASA-bashing" in the light of NASA's recent problems.

NASA's plans for EOS put as many as 16 different instruments on each of six large platforms to allow simultaneous observations of regions of interest. But a number of scientists, including James Hansen, director of the Goddard Institute for Space Studies, and members of a American Institute of Aeronautics and Astronautics (AIAA) review panel, have called on NASA officials to rethink the EOS design, arguing that the same scientific results can be achieved more reliably with the tightly-grouped "clusters" of specialized satellites similar to what the TRW report proposes.

The biggest concern is the possibility of a total loss of one of the proposed EOS platforms. All six are to be carried into polar orbits by relatively untried Titan IV rockets, whose predecessor, the Titan III, has suffered failures of varying degree in more than half of its launches over the past five years (see *Nature* 344, 281; 1990). If EOS were to be built around smaller satellites, they would probably be launched from more reliable Atlas or Delta boosters, NASA officials say.

A breakdown in a critical power, control or data-transmission component

of the EOS platforms could render all the on-board scientific instruments useless, critics point out. Shifting the emphasis to small satellites "alleviates the danger of placing all eggs in one basket [and] the possibility of losing all data from one launch or subsystem failure", Hansen writes in a paper scheduled to appear in *Issues in Science and Technology* this autumn.

With an orbiting group of several simple satellites, even the total failure of any one probe would be likely to result in no more than a partial loss of coverage. Small replacement satellites can be built relatively cheaply and quickly, the AIAA panel noted in a report released last month.

Other critics stress the danger of magnetic, electrical and thermal interference between sensitive instruments on a shared platform. And only with small satellites can researchers launch small packages of instruments to take advantage of new technology or to focus on a new area of research. Both Hansen and the AIAA panel note, for example, that because the EOS platforms will be restricted to polar orbits, they will be restricted in their ability to monitor hour-by-hour variations in clouds — a critical deficiency if clouds turn out to be as important to the global warming equation as is now believed. Indeed, Hansen's chief proposal is for additional satellites in both polar and inclined orbits to maximize coverage.

The TRW report says that many of the planned EOS instruments could make simultaneous measurements from small satellites orbiting in formation and separated by distances of three to five kilometres. TRW researchers Carl Graves, Peter Gottlieb and Alan Rosen decided that 24 main instruments of the type proposed for the first three EOS platforms near the end of the decade could be distributed on 17 smaller satellites in several clusters without loss of accuracy. The relative positions of the satellites could be maintained with only small daily adjustments, Rosen says. Because the satellites within a cluster are so closely grouped, they would be able to observe the same location on the ground within a half second of each other, which allows effectively simultaneous measurements even under rapidly changing weather conditions.

The TRW scientists are scheduled to release their report at a meeting of the International Astronautical Federation in

FOSSIL REPTILE

Lizzie home safe

London

LIZZIE, the fossil of the earliest-known reptile (see *Nature* 342, 676; 1989), now has a secure home at the National Museums of Scotland. The announcement comes after the successful conclusion of an appeal to prevent the Scottish fossil from being sold by its discoverer, Stan Wood, to the Museum für Naturkunde in Stuttgart.

Wood and the Stuttgart museum had agreed to suspend the sale until 31 July in order to give the National Museums of Scotland a chance to raise the £205,000 purchase price. A last-minute donation of £40,360 by the National Heritage Memorial Fund and another £20,000 from West Lothian District Council made up the price, following the lead set earlier this year by the £10,500 contribution from the Geologist's Association which prompted Wood to drop the price by the same amount (see *Nature* 345, 99; 10 May 1990).

This intensive wheeling and dealing, unprecedented in the quiet world of vertebrate palaeontology, has now reached a satisfactory conclusion that will allow Lizzie to be given a formal geological name. The proposal is *Westlothiana curryi*, in recognition of the fossil's place of origin and the part played by West Lothian District Council and the Curry fund of the Geologist's Association in securing the fossil for Scotland.

Henry Gee

October in Dresden, West Germany.

Shelby Tilford, director of NASA's earth science and applications division which runs the EOS programme, says that a switch to small satellites at this point would constitute a "new start" and could mean as much as a four-year delay in the project. A 1989 NASA study estimated that small satellites would cost 10–20 per cent (\$4,000–\$8,000 million) more than the planned large platforms. But the AIAA report warns that "the theoretical cost advantage claimed for large programs typically turns out, in practice, not to be realized because the amount of coordination . . . they require seems always to exceed the original projections". Big systems tend to be clumsy", it adds.

In April, an interim report from the National Academy of Sciences recommended that NASA investigate further the possibility of multiple small satellites as an alternative to its current EOS plans, at least for the second half of the project (see *Nature* 344, 578; 1990). Panel members say that the final version of the report, due out later this year, will conclude that NASA's arguments for three large platforms for the first half of the programme are "overwhelming", but that technical reasons for large — rather than small — platforms for EOS's second half are much weaker.

G. Christopher Anderson

Lighting up the sky

Washington

AFTER several delays due to technical problems, bad weather and more technical problems, the Combined Release and Radiation Effects Satellite (CRRES) was launched on 24 July by an Atlas-1 rocket from Cape Canaveral, Florida. From a highly elliptical orbit, CRRES will, over the course of a year, release puffs of fluorescent ionized gas at altitudes ranging from 240 to 21,000 miles, allowing scientists on Earth to study the structure and dynamics of the Earth's magnetic field.

The launch itself marked an important change of style for the National Aeronautics and Space Administration (NASA): instead of buying the rocket and launching it, NASA contracted out the task of putting CRRES into orbit to General Dynamics, the manufacturer of the Atlas-1. Efforts to create a commercial space business in the United States were begun by the Reagan administration after the Challenger explosion, but the first commercial launch was not until last year, when McDonnell Douglas used one of its Delta rockets to put a British communications satellite into geostationary orbit (see *Nature* 341, 5; 1989). In the future, NASA intends that scientific satellites too should be launched by commercial arrangement with the various US rocket manufacturers.

The purpose of CRRES is literally to illuminate the geometry of the Earth's magnetic field in the upper atmosphere and near space. On board are 24 canisters, each containing about 10 kg of barium, calcium, lithium or strontium along with a small charge of boron and titanium, which, once ignited, burns spontaneously to vaporize the metals and create a cloud of ionized atoms.

Fluorescing under the action of ultraviolet radiation from the Sun, the glowing ion clouds will move along magnetic field lines, betraying their structure for the benefit of researchers.

By virtue of its elliptical orbit, CRRES will be able to release tracers into both the ionosphere, the ionized upper portion of the Earth's atmosphere, and the magnetosphere, the region beyond the atmosphere that is occupied by energetic charged particles supplied by the solar wind.

Both the ionosphere and the magnetosphere are regularly disturbed by outbursts of charged particles from solar flares, disrupting radio transmissions around the Earth. These phenomena have indirectly revealed something of the physical characteristics of the ionosphere and magnetosphere, but, by allowing experiments under controlled conditions, CRRES will be able to paint a more exact

picture of these regions.

In addition to its payload of chemical bombs, CRRES also carries a number of instruments supplied by the Department of Defense to measure electromagnetic activity and particle densities in the vicinity of the satellite. These devices will be used to help choose the best moment to release the canisters, and to measure the properties of the ion clouds produced.

According to Gene Wescott of the University of Alaska, a principal investigator on several of the CRRES experiments, the ion clouds created in low-altitude releases should be visible to the naked eye for periods of many minutes. Inhabitants of the Caribbean island of Puerto Rico are likely to get the best show: in June and

July next year, low-altitude CRRES experiments will be augmented by simultaneous releases of ion clouds from sounding rockets.

In some of these experiments, a mixture of barium, calcium and strontium will be released; their different masses and ionization energies make their interaction with the ionosphere visibly different in ways that will diagnose the complex properties of the plasma.

Once the ion clouds disperse and dim, it will take months, perhaps years, for the barium and other metals to descend to the surface. And environmentalists need not be alarmed. The tens of kilograms of barium aboard CRRES are far outweighed by the many tons that drop into the Earth's atmosphere every year in the form of micrometeorites.

David Lindley

Japan's angel tours the sky

Tokyo

ON Saturday, 4 August, Japan's first lunar probe Hiten (named after a Buddhist angel) will flash past the Moon in one of a rapid succession of lunar passes which, if successful, will send the tiny satellite hurtling off on its furthest venture yet into space. The probe will then return in March for some risky 'aerobrake' experiments in the Earth's upper atmosphere.

At 182 kg, Hiten weighs no more than a Japanese Sumo wrestler. But by cleverly exploiting the gravitational fields of the Earth, Moon and Sun, engineers at the Institute of Space and Astronautical Science (ISAS) hope to make Hiten perform a complex series of loops around the Earth and Moon without completely expending the probe's very limited stores of fuel.

Launched by a small solid-fuel rocket in January, Hiten was pulled out of an elliptical Earth orbit in March by the gravity of the approaching Moon. As it swung by the Moon, Hiten dropped a mini-satellite about the size of a basketball into lunar orbit before proceeding into a large elliptical orbit around the Earth extending about 750,000 km into space.

After nearly four months of leisurely Earth orbits, Hiten again passed close by the Moon on 10 July and was pulled into a tighter Earth orbit, with an apogee of 560,000 km, that brings the probe past the Moon every month.

Following three more lunar passes on 4 August, 7 September and 2 October, the Moon will sling the probe off into space, sending it out to a distance of 1,350,000 km from Earth on 17 November before the Moon catches it again early next year and brings it back into a tighter Earth orbit.

Another series of close encounters with the Moon will then force Hiten into an

orbit that will bring it careering through the Earth's upper atmosphere at a height of only 120 km in mid-March 1991. ISAS engineers then hope to use the braking effects of the Earth's upper atmosphere to bring the high-speed Hiten back under control.

Jun Nishimura, director general of ISAS, says that if the probe survives its ride through the atmosphere it may then be placed at one of the zero-gravity 'Lagrange points', where the gravitational forces due to the Earth and the Sun cancel each other. It will be allowed to just "sit for a while", says Nishimura, after which Hiten's last drops of fuel may be used to push it into a lunar orbit alongside the mini-satellite released in March.

The small lunar orbiter has been the only disappointment of an otherwise successful mission. Shortly before the orbiter was released into lunar orbit, it was realised that the telemetry system of the orbiter was not working, so that ISAS engineers could not track it.

ISAS called on astronomers throughout Japan to train their telescopes on the Moon on 18 March, when the lunar orbiter's tiny engine was fired after it had been released by Hiten. A plume from the rocket was indeed photographed with a telescope on Mount Kiso, 200 km west of Tokyo, and although the lunar orbiter has never been seen or heard of since, ISAS scientists are confident that it successfully achieved lunar orbit.

But it is still uncertain whether Hiten will eventually join the lunar orbiter. Project manager K. T. Uesugi says the aerobrake experiments in the Earth's upper atmosphere next March are "quite risky" and may turn out to be the "end of Hiten's mission".

David Swinbanks

SUPERCONDUCTING SUPER COLLIDER

The sky is the limit

Washington

How much is too much for the Superconducting Super Collider (SSC)? At an estimated \$8,000 million earlier this year, the planned particle accelerator's cost was already larger than the Gross National Product of two-thirds of the world's nations, the entire US yearly spending on health research and the cost of all current European particle accelerators combined. Now a Department of Energy (DOE) advisory panel says that the project will need still more — difficulties with the SSC's magnets and the extra costs of any delays in obtaining funds from Congress may drive the total price to \$8,600 million by its completion in 1998, the panel said last week.

The SSC cost estimate panel, comprising members of DOE's High Energy Physics Advisory Panel (HEPAP), was the most pessimistic of three groups that have re-examined the project's financial prospects for DOE. An internal team of DOE experts puts the cost at \$8,300 million. And an estimate by University Research Associates (URA), the contractor which will actually build the SSC, promises to finish the project at the bargain price of \$7,840 million. A review by a fourth group, made up of outside financial experts, is still uncompleted. DOE will compare all four estimates and submit a final report to Congress on 17 August.

The chief concern seems to be the schedule and technical challenges of building the superconducting magnets. "The project has been planned on an extremely optimistic schedule, with every event being 'head-to-tail' with others... [S]uch an approach results in an unrealistically low estimate, which will inevitably be overrun", HEPAP warned. The panel also noted that the SSC has had difficulty attracting staff.

Beyond technical problems looms the very real possibility that Congress, squeezed by deficit reduction and still uneasy about the scientific arguments behind a 54-mile superconducting accelerator, may cut the project's funding at some time during the next eight years. Moore said that a congressional funding delay could add over \$50 million a month to the SSC's final cost. The HEPAP panel says that schedule delays are likely to contribute \$300 million to the overrun.

HEPAP also recommended that another \$300 million — which would raise the total cost to \$3,900 million — should be budgeted to build two large detectors that are part of the original SSC plan. But rather than raise the cost further, SSC officials have decided to drop the two large detectors for the time being. Two less expensive detectors — one medium, the other small — should be sufficient for the early years, says acting project manager

Ted Kozman. He says that there may not be enough physicists available to justify the operation of larger detectors when the SSC begins operation at the end of the decade. Expansion room will be built into the SSC to allow larger detectors to be installed later when the demand rises, Kozman says.



Speaking at a press conference last week, DOE deputy secretary W. Henson Moore said that the department intends to go to Congress with the URA figures, rather than the higher estimates. "We're going to hold the contractor to \$7.8 billion and to finish by 1998", he said. Although there is no penalty if URA is then forced to raise the cost, Moore said the contractor would have to document the need and get DOE approval. Submitting one of the higher estimates to Congress, Moore said, would only be sending the signal that congressional and technical delays are anticipated and compensated for. "If we were to take any other position, we'd be telling the contractor, 'We expect delays, don't worry about it,'" he said. So far this year, Congress has treated the project relatively well, granting the full DOE request of \$318 million for 1991. But until legislators trim spending across the board later this year to reduce the federal deficit, final budgets for all projects — including the SSC — will remain at risk.

Moore also announced the release of the formal request for proposals to build the superconducting magnets. Because the magnets will represent a substantial advance in technology and engineering, the companies or countries that provide them stand to become world leaders in the fledgling superconductor industry. Although DOE's offer stipulates that at least 50 per cent of the contract must go to US industry, companies from foreign countries may bid on the other half of the contract, perhaps as part of an 'in kind' contribution from their nation's government. So far, only Korea and India have expressed interest in contributing to the project.

G. Christopher Anderson

ANTARCTIC RESEARCH

Keeping it clean for science

São Paulo

THE twenty-first meeting of the Scientific Organization on Antarctic Research (SCAR) ended here last week with the addition of four new full members — Spain, South Korea, Finland and Holland — and a new associate member — Colombia — bringing the total membership to 29 countries.

The scientific focus of the meeting was the effort to integrate Antarctic research with global scientific concerns, particularly climate change. The threat to the region's environment was also a theme of the meeting: "The environment is not one major issue, it is *the* major issue", said Brazilian geologist Antonio Carlos Rocha-Campos, executive secretary of SCAR and chairman of the meeting.

Dealing with the waste produced by an Antarctic population that can reach 4,000 in the summer months (December to March) has become one of the greatest environmental concerns. SCAR has produced a rulebook on waste management, but enforcing it is another matter. Not all of the 50 Antarctic stations have the requisite waste-disposal technology, but

Rocha-Campos diplomatically refused to name the worst offenders. Inspections conducted by Greenpeace, however, have documented open-air trash burning at the Chinese, Chilean, Argentine, Uruguayan, US and Soviet bases.

Tourism is also becoming a headache, both as an additional source of pollution and because of disruption to the scientific activities of the stations. SCAR, an advisory body to the Antarctic Treaty nations which coordinate research efforts, considers tourism a "legitimate activity" when properly ordered, but scientists are finding it troublesome.

SCAR has no power to enforce its recommendations but has, for example, suggested that unnecessary duplication of activities should be avoided, especially in crowded areas such as the Antarctic Peninsula and the nearby King George Island. Crowding exacerbates environmental problems, but the mood of the SCAR meeting was that it is the responsibility of scientists to deal with problems that will ultimately disrupt the science they have come to Antarctica to conduct.

Ricardo Bonalume Neto

First experiment approved

Washington

AFTER bringing in their colleague Claudio Bordignon from Milan, Italy, to present the results of animal tests, R. Michael Blacse and W. French Anderson of the National Institutes of Health (NIH) have succeeded in convincing a subcommittee of the NIH Recombinant DNA Advisory Committee (RAC) that the time is right to go ahead with the first human gene therapy experiment. The researchers hope to treat children with severe combined immunodeficiency caused by ADA (adenosine deaminase) deficiency — a rare inherited immune disorder affecting fewer than 20 people worldwide — and although the experiment must still be approved by the RAC, the director of NIH and the US Food and Drug Administration, Anderson is optimistic that clinical trials in four children could begin as early as mid-autumn.

With increasing numbers of experiments being submitted for approval — there are four in the pipeline — there is concern that the review system will not be able to cope. The subcommittee is trying to carry out “a very critical sentence by sentence review”, says Anderson, who feels that “this depth of review is not necessary”.

All the new proposals awaiting review by the subcommittee are based upon the first gene transfer experiment, approved in January 1989 (see *Nature* 337, 294; 1989), which was designed to trace the movement of tumour-infiltrating lymphocytes (TIL) in terminally ill cancer patients. The experiment was very controversial when first proposed, but has since formed the cornerstone of all new gene transfer and gene therapy proposals. In what are referred to as the N2-TIL experiments, Steven Rosenberg of the National Cancer Institute (NCI) isolated TIL cells from a patient's tumour, marked them with a gene for neomycin resistance and readministered them to the patient. Although the gene conferred no therapeutic benefit on the patient, it allowed researchers to track the path of TIL cells in the patient's body and determine whether the cells ‘homed’ to the tumour to fight the cancer.

With the safety of the N2-TIL gene transfer established, Blacse, of the NCI, and Anderson, of the National Heart, Lung and Blood Institute, plan to move on the “gene therapy” by inserting genes of therapeutic value into children who suffer from ADA deficiency. Using the methods of the N2-TIL experiments, but substituting the patient's own T lymphocytes for TIL cells, researchers hope to correct the ADA deficiency by inserting a normal human ADA gene into the T lymphocytes and returning the gene-

corrected cells to the patient.

Of the four new experiments in the pipeline, two almost identical gene transfer experiments have been submitted by K. Brenner of St Jude's Children's Research Hospital in Memphis, Tennessee, and K. Cornetta of the University of Wisconsin. Brenner and Cornetta hope to determine the mechanism of bone marrow reconstitution following autologous bone marrow transplantation (ABMT), in which the patient's own bone marrow is used to treat leukaemias and some solid tumours. Marrow cells can be marked using the N2-

PEST CONTROL

Cleverer ways to kill?

Washington

ATTEMPTS by Western nations to combat the huge plague of locusts and grasshoppers that struck Africa in the late 1980s relied far too much on brawn and far too little on brains, according to an unusually critical report* released from the US Congressional Office of Technology Assessment (OTA) earlier this week.

In a massive campaign led by the US Agency for International Development (AID), some 13 million litres of insecticide were sprayed over 100,000 square kilometres of northwest and northern Africa between 1986 and 1989. As a result, experts claimed, a locust plague was for the first time stopped in its tracks by human intervention. But OTA argues that it is not at all clear whether it was the insecticide or just fortuitous changes in the weather that wiped out the insects.

Rather than taking credit for ending the plague, the report says that AID officials should be taking responsibility for “the mostly uncounted health and environmental costs of insecticide-based control programs; redirecting development funds to emergency efforts; and focusing on a few insects that, while highly visible, do not cause crop losses as great as some other agricultural pests”. In 1986, OTA contends, donors spent \$40 million and succeeded in saving only \$46 million worth of crops.

A better approach, the OTA says, would accept that most locust upsurges do not result in famine. Instead of major assaults on this one enemy, what are needed in Africa are intelligent integrated pest management and monitoring systems for insects, weather and vegetation. Such an approach would deal with different pests according to their economic impact, would stress prevention over crisis management and would look for long-term development of African agriculture over

TIL gene transfer system, and tracked when returned to the patient.

Building on his earlier N2-TIL experiments, Rosenberg is hoping to improve the anti-cancer properties of TIL cells by inserting a gene coding for tumour necrosis factor (TNF) — an experimental anti-cancer drug — into TIL cells. In a similar vein, M. T. Lotze of the University of Pittsburgh is looking at ways to improve the targeting and efficacy of TIL cells in patients with malignant melanoma. Performing an identical experiment to the N2-TIL gene transfer study, Lotze plans to study the effects of administering Il-2 and Il-4 in conjunction with TILs.

Diane Gershon

emergency intervention.

The report has already provoked a strong response. Senator Patrick Leahy, chairman of the foreign operations subcommittee which funds the AID's locust control programme, described the massive use of insecticides as “absolutely unacceptable” when it costs as much to protect the crops as the crops are worth, let alone health and environmental costs.

Locust experts are united in their support for longer-term integrated pest management, but their views of the 1980s campaign make the OTA report seem too combative. Joyce Magor, head of the department of pest ecology at the UK's Natural Resources Institute, points out that a locust plague is usually stopped by a combination of events: “unusual migrations that send a proportion of the insects to a place where they cannot breed successfully, lack of rain in locust breeding areas and the coincidental arrival of locusts in countries where control is effective”. In 1988, a fortuitous air current sent huge swarms of locusts on a final voyage out over the Atlantic, while some well-organized campaigns mopped up huge numbers of locusts. Neither weather nor human intervention alone can expect to take the credit for ending the plague.

Measuring the effectiveness of a campaign in terms of how much damage it averted is a “guessing game” says Magor, “because you would have to estimate where you killed them, and where they would have gone if you had not.” In the 1980s plague, swarms crisscrossed northern Africa but spent most of their time in subsistence farming areas. They did not enter the regions of Morocco, for example, where crops are grown to supply out-of-season specialities to Europe and large financial losses might have been recorded. Had human intervention prevented crop loss there, OTA's sums might have added to a different conclusion.

Alun Anderson

* A plague of locusts — special report. OTA-F-450 (Washington DC: US Government Printing Office, July 1990).

UK RESEARCH SPENDING

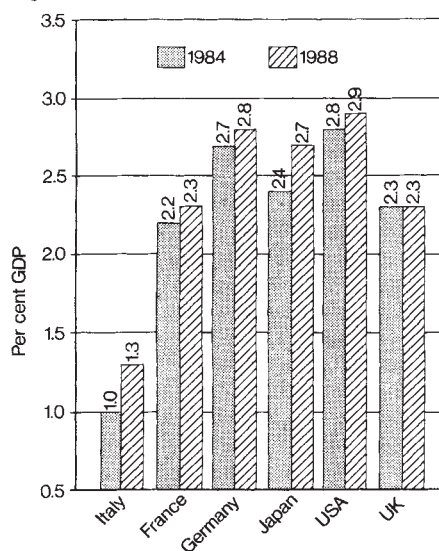
Underfunding hits patents

London

BRITISH investment in research and development is failing to match that of other nations and research output, measured by the number of patents granted in the United States, is suffering accordingly. Those are the key statistics buried in the 300-plus page *Annual Review of Government Funded Research and Development*, published by the UK Cabinet Office.

A total of £10,300 million was spent on research and development in the United Kingdom in 1988, representing an increase in real terms of 2.1 per cent from 1987. Thirty-seven per cent of funding came from government, 51 per cent from British industry and a small fraction from overseas.

But if the figures are converted to a proportion of the UK's gross domestic product (GDP), as is usual for international comparisons, British spending is seen to have stagnated at the 1985 level. In five other countries surveyed, research and development spending as a proportion of GDP is increasing (see figure), leaving Britain behind West Germany, Japan and the United States, but ahead of



Gross domestic expenditure on research and development in six countries. West German and UK figures are for 1985 and 1988. Japanese figures are for 1984 and 1987.

Italy and level with France. Even this analysis may exaggerate the British research and development effort: a recent report from the House of Lords Science and Technology Committee suggested that much of British defence research is not truly innovative and so does not conform to internationally accepted definitions of research and development (see *Nature* 345, 5; 3 May 1990).

For the first time, the Cabinet Office has included data on patents as a measure of the output of British research and development. The study looked at patents

granted in the United States in order to avoid bias when making comparisons with European countries and Japan.

Britain was granted 14 per cent more US patents per capita in 1984–88 than in 1979–83. But other countries did better. West Europe was up 23 per cent on average, led by West Germany with 28 per cent. The Japanese performance eclipses that of Europe, with 79 per cent more US patents per capita granted to Japan in 1984–88 than in 1979–83.

The poor British performance is part of a long-term decline. In 1963–68 Britain was granted more US patents per capita than the West European average, with the Japanese rate less than a quarter that of the United Kingdom. But the UK rate for 1984–88, at 44.06 US patents per million population, was almost identical to the 1963–68 figure. In contrast, the West European average rose to 58.42 per mil-

JAPAN'S SPACE PROGRAMME

Rocket of falling ambitions

Tokyo

JAPAN'S next-generation H-II rocket will have less power than expected if and when it blasts off on its first flight in 1993.

The National Space Development Agency (NASDA) announced last week that the thrust specifications of the main engine of the rocket have had to be reduced so that the agency's engineers can meet the 1993 launch deadline.

The Japanese-made liquid fuel engine in the first stage of the H-II has been plagued with problems ever since test firings began last year. In the first test runs, the turbine blades of the liquid hydrogen pump resonated and cracked, forcing a one-year delay in the first test launch of the rocket (see *Nature* 340, 253; 1989). In subsequent tests, the engine burst into flames.

After a series of 16 test firings between March and May this year, NASDA engineers thought they had their problems solved. But earlier this month the engine again caught fire during a test run.

This time the fire had an entirely different cause. M. Miyazawa, director of NASDA's propulsion systems group, says the fire began when hydrogen leaked from a small pipe feeding the liquid fuel to the ignition system of the engine. The pipe cracked when NASDA engineers deliberately made the engine vibrate to collect data necessary for the design of a vibration suppression device which will be incorporated into the engine.

Miyazawa stresses that under normal circumstances the engine would not have caught fire. But NASDA will now redesign three pipes in the engine, including

lion and Japan was granted 114.62 US patents per million of its population.

There are some seeds of comfort for the British scientific community. The number of academic research staff in UK universities has risen steadily over the 1980s, from 6,309 in 1983–84 to 8,413 in 1988–89, and industrial research and development spending is at a record level, at £6,900 million in 1988.

Projections for UK spending up to 1992–93 may already have been overtaken by events. A decrease in the amount of civil research and development, relative to spending in the defence sector, is forecast. But the figures are based on the government's annual statement on public expenditure made last autumn, before the political upheavals in Eastern Europe raised the possibility of a 'peace dividend'. A predicted decline in total research and development spending in real terms may also be found incorrect when the public expenditure statement is released in the autumn.

Peter Aldhous

the one that broke. And, more important, NASDA engineers now realize that they cannot build a reliable engine that meets the original thrust specification of 120 tons (in vacuum) by 1993. So instead, the thrust will be reduced to 110 tons.

Naotaka Oki, director of the space development division of the Science and Technology Agency (STA) to which NASDA is affiliated, emphasizes that even with the reduced thrust the H-II will still be able to meet its original goal and place a 2-ton payload into geostationary orbit. That will be possible because the power specifications of the solid fuel booster rockets and second-stage engine were increased last year. But the rocket will not be able to lift 2.2 tons as was hoped when the new specifications were announced, Miyazawa says.

When the rocket is used for the double launch of a geostationary meteorological satellite (GMS-5) and an unmanned space platform (SFU) in 1994, extra solid fuel boosters will be added to the first stage. But this is a "one-off case" and, despite reports to the contrary in the Japanese press, extra boosters will not be required for other launches, Oki says.

The H-II's first-stage liquid fuel engine, like that in the US Space Shuttle, uses a staged combustion cycle that provides higher performance than the gas-generation cycle applied in other liquid-fuel rockets such as Ariane and Delta. But this type of engine is notoriously difficult to develop. Miyazawa admits that, even with the reduced thrust specification, meeting the 1993 launch deadline will be "tight".

David Swinbanks

Reactor judged not so fast

London

ANY lingering hopes held by the UK Atomic Energy Authority (UKAEA) for a revival of Britain's flagging fast-breeder reactor programme were dashed last week by the publication of a report from an all-party House of Commons committee. The report agrees with the government's decision to end support for the prototype fast reactor at Dounreay, Scotland, from 1994 and concludes that continuing British investment in a European Fast Reactor (EFR) after construction begins in 1997 is unlikely to be worthwhile.

Fast reactor research has consumed £4,000 million (in 1988 money) of government funds since 1954. When development began, fast reactors made sense because it was thought that uranium fuel would eventually be in short supply. Fast-breeder reactors can extract 60 times more energy from a quantity of uranium than conventional thermal reactors. But there is still no shortage of uranium.

The energy select committee rejects claims from the old Central Electricity Generating Board (CEGB) that fast reactors are needed as part of Britain's strategic energy planning. Major expenditure on fast reactors can be justified only if there is "a potential economic case", and calculation of the likely future supply of uranium show that it is very unlikely that fast reactors will become economic before 2020. Further development should wait, the committee says, because of the risks of designs "becoming obsolete" before they are required commercially.

Former energy secretary Cecil Parkinson decided in 1988 that funding for the UKAEA Dounreay fast reactor should end after 1994. But Britain is still involved

with France and West Germany in a plan to begin building the European Fast Reactor in 1997.

British experience with the Dounreay reactor is valuable to the project, but the United Kingdom is a junior partner financially, contributing some £10 million a year — about 15 per cent of the total. The remainder of the research and development costs are divided roughly equally between France and West Germany. But the UK contribution is expected to rise to almost £350 million in each of the two peak years of construction.

The energy committee concludes that this investment should be reviewed in 1993 and 1997, and seems at present to be unnecessary. The CEGB and UKAEA argued that British withdrawal from the EFR would wreck the collaboration, but the committee believes that France will probably continue with fast reactor development in any event. Jean Leduc, from Novatome, a French company which builds nuclear power stations, says fast

NUCLEAR SAFETY

reactor development is "more urgent" in France than the United Kingdom. France already relies heavily on nuclear generated electricity, and has a generation of pressurized water reactors that must be replaced between 2010 and 2020. Leduc says the EFR programme is organized to produce a commercial design by this time.

In the future, concern over global warming may change energy policy and favour power plants that produce little carbon dioxide. But the committee did not feel these considerations would affect the economic viability of fast reactors. Renewable forms of electricity generation, such as wind, wave and tidal power, are in the same situation as fast reactors — "technically proven but not [yet] commercially viable" — and other forms of nuclear electricity generation, notably pressurized water reactors, share the advantage that they do not produce carbon dioxide but are cheaper, unless the price of uranium increases substantially.

The report says that large-scale research and development projects should be kept under "close and continuous review".

Peter Aldhous

Kicking the habit

Munich

LIKE a person who has recently given up smoking, non-nuclear Austria is trying to persuade its neighbours that they too should give up nuclear power, for their own sake and for the sake of those around them.

Concern about the safety of some of Czechoslovakia's oldest power plants has provoked Austria's latest antinuclear crusade. Last week, Austria offered to sponsor an inspection of the two oldest reactor blocks at Bohunice, in Slovakia near the Austrian border, and to deliver electricity valued at 10 million schillings (about \$830,000) a day for up to 10 days during which the plant will be shut down.

Austria chose to abandon its own nuclear programme in 1987, but construction and operation of nuclear plants in three neighbouring states — Czechoslovakia, Hungary and Yugoslavia — has rolled merrily along (see *Nature* 346, 98; 12 July 1990). Although the Czechoslovak government ultimately agreed to allow Austrian experts to join in a safety inspection of Bohunice by 10 August, new Czechoslovak Environment Minister Josef Vavrousek says that "there is a 99 per cent chance" that such an inspection would find the reactors safe. Austrian Environment Minister Marilies Flemming disagrees, saying that if even a fraction of the reports she has heard about Bohunice are true, it must be shut down immediately.

Flemming will travel to Hungary next week, where a spokeswoman said she

would urge the Hungarians to resume construction of the huge Nagymaros hydroelectric dam project, which the Hungarian government stopped last year in order to protect the Danube and surrounding areas. Electricity generated by the project would provide an alternative to that from nuclear power plants. After that, she will try to persuade Yugoslavia to shut the Krsko nuclear plant, which is in Slovenia, also not far from Austria.

Beyond the goodwill gesture of replacing lost power for a few days, Austria has made few offers to help Czechoslovakia finance the building of new power stations. The Austrian government estimates that it would cost \$16,000 million just to bring existing Czechoslovak power stations up to Western standards. Austrian ministers met Western European leaders in Salzburg last week to try to persuade them to set up an "Energy Marshall Plan" for Eastern Europe.

A spokesman for the International Atomic Energy Agency (IAEA) in Vienna, which will carry out a limited safety inspection of its own in October, said it takes the problems with reactors of the type found in Bohunice (VVER 440-V 230) "very seriously". Such reactors are also found in Bulgaria, the Soviet Union and also in East Germany, where they were judged unsafe and ordered shut earlier this year. IAEA spokesman David Kyd said it would try to "get everyone around a table" to discuss the future of these reactors.

Steven Dickman

OCEANOGRAPHY

UFC funds deep-sea centre

London

The UK Universities Funding Council (UFC) has agreed to provide £10 million for a new centre for deep-sea oceanography in Southampton. The centre will open in 1994 at an estimated cost of £43 million, mostly supplied through the Natural Environment Research Council (NERC). The UFC money is to allow researchers from the University of Southampton to move to the centre and work with NERC scientists.

The centre will have an important role in climate change research, hosting the UK contribution to the World Ocean Circulation Experiment, a large project investigating the relationship between oceans and climate — a major uncertainty in current climate models.

Peter Aldhous

WEST GERMAN RESEARCH

Greens to shut Berlin reactor

Munich

OPponents of nuclear energy in Berlin are poised to shut down a research reactor and are threatening the future of an important research institution, the Hahn-Meitner Institute (HMI). HMI, named after Otto Hahn and Lise Meitner, who together with Fritz Strassmann discovered nuclear fission in Berlin in 1938–39, is the only West German national laboratory (*Grossforschungseinrichtung*) in West Berlin.

Last week, Environment Senator Michael Schreyer of the *Alternative Liste*, the local branch of the environmentalist Green Party, refused to sign a licence for the restored research reactor at the Hahn-Meitner Institute. Ironically, she does not contend that the reactor itself presents a danger, but rather that the disposal of radioactive waste from the reactor is not and never will be assured as long as West Germany has no facilities for waste reprocessing and disposal.

The newly refurbished 10 MW research reactor was meant to be used as a neutron source for biology, medicine and chemistry as well as physics, and was completed at a cost of DM170 million (about \$110 million) late last year. Since then, it has been consuming DM1.8 million a month in maintenance costs.

West German Research Minister Heinz Riesenhuber (Christian Democrat), whose ministry provides 90 per cent of the HMI budget, has threatened to withdraw support from HMI if the reactor is not licensed by 31 July. According to Hans Stiller, scientific director of HMI, if the reactor licence is denied, then there will be an exodus of researchers from the institute that could ultimately force it to close down.

Schreyer's refusal to license the reactor could be reversed when a new government is elected in West Berlin later this year, says Stiller, but such a move would certainly be challenged in court and the reactor would have to remain shut down in the meantime. Then the exodus of researchers would begin in earnest, he fears, because the wheels of justice turn "very slowly" in West Germany.

The only chance to save HMI, says Stiller, is for West Berlin Governing Mayor Walter Momper (Social Democrat) to remove from Schreyer the task of licensing the reactor. Stiller admits that this tactic would certainly bring down the fragile 18-month-old government coalition between the Social Democrats and the *Alternative Liste*.

Nevertheless, there are compelling reasons for Momper to risk the coalition in order to save HMI. Momper's Senator for Science, Barbara Riedmüller-Seel, writing in the West Berlin daily *Der*

Tagesspiegel, cites the reactor's "strategic importance" for science in West Berlin. If the HMI reactor is not licensed, she argues, the city will lose out when new research institutions are created. At risk would be the electron storage ring BESSY II, another politically controversial project opposed by *Alternative Liste*.

Momper has risked the break-up of the coalition on other issues recently, in anticipation of new elections encompassing both West and East Berlin. *Alternative Liste* support is bound to wither in the reunified city, where economic concerns will dominate over environmental ones. But Momper will have to act fast if he wants to dismiss Schreyer before her decision not to license the reactor is made final. She told HMI that it would have only until 6 August to raise objections to her decision.

Although the issue has dragged on ever since the Social Democratic-*Alternative Liste* coalition took power in West Berlin, it continues to mobilize the West German research community. In July, the chairman of the West German national laboratories, Harald zur Hausen, appealed to both Riesenhuber and Momper to prevent any further delay in licensing the reactor.

Stiller and the Social Democratic majority in the West Berlin Senate contend that waste disposal for the HMI reactor is already assured. HMI last year signed a contract with the British reprocessing facility at Dounreay to reprocess its waste, return the restored fuel rods, and leave the remaining waste there for 29 years, by which time West Germany will have found a place to put its own nuclear waste.

According to Stiller, West German law requires only six years of guaranteed storage, so the current HMI arrangement exceeds the legal minimum by 23 years. But Schreyer claims that transport over such long distances is too risky. West Germany needs a plan for long-term waste disposal now, she says, or else the operation of any nuclear reactor is irresponsible.

The repercussions of Schreyer's demand may reach far beyond the future of HMI, Stiller warns. There are other *Länder* with Social Democratic governments that would be only too happy to challenge the federal government in court over the issue of waste disposal.

The new Social Democratic-Green government in Lower Saxony has announced that it plans not to continue construction at Gorleben and Konrad, which were intended to become West Germany's only sites for final storage of high- and medium-level wastes respectively.

Steven Dickman

SCIENCE JOURNALS

Parlez-vous français?

Paris

IN an attempt to halt the decline of French as a scientific language, the Ministry of Research and Technology and the National Institute of Health and Medical Research (INSERM) are offering two FF400,000 (\$72,000) prizes for the best original papers published in French in the journal,



Comptes rendus de l'Académie des Sciences. Now that the *Annals de l'Institut Pasteur* is published in English, *Comptes Rendus* is the most respected science journal to be published in French — albeit with long abstracts in English.

Over the past decade, French scientists have gradually abandoned native journals in favour of international English-language journals. Recently, researchers in life sciences at CNRS, the national science research centre, were more or less told by their controversial director, Claude Paoletti, that only such publications would have any weight when it came to laboratory assessments for grants — a move now rather at odds with the ministry.

The new effort to improve the image of French language publications is also an act of defiance. There is a common feeling here that papers submitted to international journals are often rejected because the researchers are not American or English.

Peter Coles

INDIA

Forest cover disappears

Bangalore

INDIA has lost 1,907 sq. km of forest cover in the past four years according to the results of a new assessment made by the Forest Survey of India using Landsat data. An earlier assessment based on satellite imagery collected between 1981 and 1983 showed that the country then had a forest cover of 642,041 sq. km. By 1985 to 1987, the period on which the new assessment is based, forest cover had declined to 640,134 sq. km. Mangrove forest cover increased slightly from 4,000 sq. km to 4,200 sq. km. According to India's Ministry of Environment, steps being taken to slow deforestation are provided by the National Forest Policy and Forest Conservation Act of 1988 and include a centrally sponsored scheme to encourage the use of fuels other than wood.

Radhakrishna Rao

Geologists' Association response

SIR—Des Griffin in his letter¹ on the rumour over the Natural History Museum's corporate plan is quite categorical. "The problem is the British government and its continued refusal to recognize the enormous importance of museums in all areas of science and education." He asks how the Geologists' Association would approach the problem — "presumably 132 years have taught them something". Quite so.

First, it is evident that if government does not recognize the importance of museum research, it is because it does not understand it. If it does not understand, it can only be because no-one has taken the trouble to explain it in language it can understand. The responsibility lies with the scientific community, not the government.

I should like, as president of the Geologists' Association, to outline the role it has played. The crisis over support for taxonomic research at the Natural History Museum is part of a much broader issue, as Griffin clearly perceives. All governments find it difficult to justify extensive financial support for long-term strategic research, data-gathering and geological surveying as well as taxonomy of animals, plants and fossils. Pure or 'blue skies' research and applied research they can grasp.

Whenever the funding of research has been discussed by bodies such as the Centre for Policy Studies, I have stressed the need for adequate support of such long-term data-collecting research.

In October last year, the Institute of Economic Affairs (IEA) Education Unit (a think-tank with the reputation of originating government economic policies) held a conference on the funding of research², whose purpose was to formulate a policy for consideration by government ministers. The conference was concerned not with political issues but with finding the best and most effective way of supporting research.

I spoke on the problems of funding long-term strategic research, using as examples the work of the Geological Survey and the Natural History Museum, and my case was incorporated in the final conclusions of the conference:

"There is a whole area of research devoted to data collection. Some describe this as 'strategic research' . . . Examples of data collecting research are the Natural History Museum with its wealth of data, and continuing collection of data, about all aspects of the animal kingdom. Such data collection, be it the examination of different species of fly or the varying composition of the atmospheric gases above the pole, may seem to have no immediate or obvious application, but such data, in all areas of science, is fundamental to research in those

areas. Such data collection forms the 'dictionary' of the subject upon which other researchers can build.

"This area of research risks being neglected in our funding arrangements. It has to be funded with taxpayers money, nobody else today will fund it, and needs to be funded through the research councils or specifically and directly by the government.

"Even this area of research does produce an important economic return, but such is in general at one remove, it is usually not direct and not immediate."

It was at this point that the museum's corporate plan, with its deliberate decision that the scientific side should bear the brunt of the cuts, was launched on an unsuspecting world. In letters to the press, interviews and subsequent articles³, I have drawn attention to the advice proffered to the government by its own think-tank.

As president of the Geologists' Association, I wrote to the prime minister and several of her ministers, to every member of the museum's trustees and to presidents of other learned societies. The last resulted in a joint letter to the press, requesting that the scientific redundancies be held in abeyance until the government's science-funding policy had been finally decided, on the basis of the IEA deliberations.

Meanwhile, I appealed to all members of the Geologists' Association to protest to the government and to their own Members of Parliament. The minister responsible was left in no doubt as to the deep feelings regarding the imminent demise of major areas of geology and palaeontology.

Time will tell whether or not our strategy was the correct one. I leave it to your readers to judge whether Beverly Halstead has "done little to help the museum by his recent comments".

BEVERLY HALSTEAD
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1. Griffin, D. *Nature* **346**, 99; (1990).
2. Sexton, S. (ed.) *The Funding of Research* (Institute of Economic Affairs Education Unit, London, 1990).
3. *Daily Telegraph* 27 April; *Times* 27 April; *Guardian* 28 April; *Science* 11 May; *Guardian* 11 May; *Independent* 14 May (1990).
4. *Independent* 25 May (1990).

SIR—Des Griffin's letter confuses me. I am not sure whether he supports Neil Chalmers, the director of the Natural History Museum, perhaps from the viewpoint of a museum director himself, or whether he is critical of the museum's corporate plan, and if so, on which grounds.

He accuses me of ignorant nonsense. But Griffin's musings (they are not logical

arguments) do betray a touching political naivety, suffused with an inability to distinguish between the building called "the Natural History Museum", the existence of which is not at risk, and the functions within that building, which are not only at risk but are being destroyed at this very minute.

His senior-statesmanlike plea for joint pressure on the government presupposes that there is time. There is no time; staff morale is non-existent, staff are leaving even if their names are not on the redundancy list. The staff's request for reallocation of redundancies and jobs, published in the same issue as Griffin's letter, is inadequate.

I have no realistic solution to offer. Money is one problem, both now and for the future. The British government has not shown itself to be sympathetic to any organization that promotes public service as opposed to private profit. Like all governments, in the absence of internal motivation, the only thing it responds to is public pressure. These draft letters which Griffin disparages are part of this public pressure. The other problem is the attitude of management. Both problems need to be tackled at the same time. Considerably more public pressure is needed both from professionals and from the public for whose benefit the museum and its research staff exist.

There is a possible final solution which would perhaps meet with Griffin's approbation. It is not inconceivable that the collections and those research staff still remaining could be moved out of London and the museum in all senses handed over to Disneyland to do with as it pleases.

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Creating havoc

SIR—I was interested in the close juxtaposition in *Nature* for 5 July of the reports of the flaw in one of the Hubble Space Telescope's (HST) mirrors (page 3) and the letter from Christopher Lote (page 10) on scientists' view on the existence of God.

It is my understanding that one of the possible uses of HST was to throw light on the creation of the Universe. Perhaps God does not want us to know.

GILBERT F. RICHARDS

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Correction

The author of the first letter headed "Manned mission to Mars" in the issue of 28 June (*Nature* **345**, 760; 1990) is N. H. Horowitz. We regret having misspelled his name. □

Bicycling about to be explained?

A new calculation seems to have made the behaviour of bicyclists explicable — but there is a long way to go before the matter will be satisfactorily understood.

THE essential problem of the bicycle — why does a moving bicycle not tip over more often? — may at last have been solved.

That at least is the implication of a report by G. Franke, W. Suhr and F. Riess from the University of Oldenburg (*Eur. J. Phys.* **11**, 116; 1990). But one should be cautious. In the long history of mechanics, claims that the problem of the bicycle has been solved have been regularly followed by demonstrations that the claims are based on over-simple approximations that invalidate the conclusions. Ominously, even Franke and his colleagues have simplified the problem by supposing that the rider steers the bicycle only by adjustments of the position of his or her centre of gravity. It is hands-off riding. On the face of things, the solution does not accommodate what common observation demonstrates — that riders pedalling find it advantageous to make energetic movements from side to side while playing with the direction of the front wheel.

Franke and his colleagues are forgiving about previous attempts at the problem, which they acknowledge to be difficult.

They also take note of earlier demonstrations that one of the crucial determinants of stability is the distance between the axis of the steering column and the centre of the front (steerable) wheel, called the 'trail length' in the argot of the discipline. This is why the front fork of a bicycle, the device that supports the steering mechanism and the front of the bicycle frame on the front wheel, is usually bent forward towards the bottom. Their objective is to solve the problem of the moving bicycle with as few limitations as they can manage. Among other things, for example, they have allowed for wheels of unequal radius, which means that their solution should be as easily applicable to penny-farthings as to modern designs.

Much of the interest in the new attempt at the problem is the manner in which the authors have formulated it. At least superficially, it is not as complicated as might be expected.

Crudely, if a bicycle (with its rider) were a rigid body, it would have six degrees of freedom (three translation degrees of freedom and three rotations). But bicycles are not rigid: the front wheel can be steered (through a 'steering angle') while the two wheels can rotate. So there are nine degrees of freedom altogether — six intrinsic to a rigid body and three strictly internal degrees of freedom. The con-

figuration of the bicycle at any time could in principle be defined by specifying nine variables. If the bicycle is moving, for each degree of freedom there will be a coordinate in a suitably chosen reference frame and a corresponding velocity.

But it is clear that not all possible motions correspond to stable reality. To achieve that, the two wheels, for example, must remain in contact with the ground. So the problem of the moving bicycle is that of the motion of a rigid body which is subject to what are called constraints. The simplest way of dealing with such problems is to choose a set of coordinates that will automatically ensure that the constraints are satisfied, which sometimes requires a little ingenuity.

But Franke and his colleagues make the task seem simple. Of the nine configurational degrees of freedom, for example, two are eliminated by the requirement that the wheels should be on the ground, while — at least on level ground — neither the absolute degree of rotation of the two wheels nor the absolute position and direction of the frame can be relevant to stability.

The result is that the configuration of a bicycle can be described by only two variables, taken as the steering angle and the lean angle. Of course, the dimensions of the actual bicycle enter as parameters — Franke and his colleagues use the radii of the two wheels, the trail length, the distance along the perpendicular between the centre of the rear wheel and the steering axis and the distance between the centre of the front wheel and that same perpendicular as the parameters that specify the bicycle. By similar arguments, the dynamical degrees of freedom are reduced to three — the time derivatives of the steer angle and the lean angle and the scalar velocity of the bicycle (measured, for the sake of definiteness, at the rear wheel).

Turning this description of a moving bicycle into a set of equations of motion that might be solved is the more tricky part of the calculation. For practical purposes, the authors used a moving system of rectangular coordinates whose origin is the point at which the steering axis meets the ground, but whose unit vectors are along the direction of motion of the rear wheel, along the point of contact at the rear wheel with the ground to its centre and towards the hub of the rear wheel. (The last choice leads to the convenient result that the lean angle does not appear in the equations of

motion.) Even so, the outcome is a vector equation (equivalent to three simultaneous scalar equations) that cannot be solved analytically, only numerically. No doubt these equations will soon be known by heart in the places at which bicycles are designed; they are a potentially endless resource for bicycle-makers.

The immediate benefits of the investigation, unfortunately, are more a spur to further investigation than a revelation in themselves. Franke and his colleagues have allowed for the bicycle rider to adjust his position laterally relative to frame. The rider's weight is supposed to be an unladylike 70 kg. One set of solutions to the equations of motions arises by supposing that a no-hands bicyclist of this size travels in a repeating circle, with a constant steering angle and a constant velocity.

Indeed, as one would expect, the greater the lean angle, the greater must be the steering angle (for a fixed velocity) or the greater the velocity (for a fixed steering angle). It is more surprising that while, above a certain critical velocity, there should be only one combination of steering angle and rider displacement that will keep the bicycle travelling in a circle at a prescribed lean angle, below that critical speed, there are two lean angles at which the same rider displacement will keep the bicycle in a circle. Those who ask whether this is a sign of incipient bifurcation to chaos will not be surprised to be answered in the affirmative.

Perhaps the most practical implication of the calculations so far carried out is that the speed at which circular motion can be stable is sensitively dependent on the moment of inertia of the wheels: the smaller the combined moment of inertia, the greater must be the velocity. Similarly, the trailing distance crucially determines the range of velocities over which motion in a circle can be stable; the greater the forward displacement of the front wheel, the greater the range of stable velocity. That, of course, is what all circus bicycle-riders know by instinct.

What all this means for real bicycle-riders is hard to tell. That the forward displacement of the rider's centre of gravity is the equivalent of lengthening the trailing distance is unsurprising.

This, no doubt, is why riders stand on the pedals when going very slowly. But what the new equations of motion mean for the behaviour of bicycle-riders remains to be determined. **John Maddox**

Passing electrons one-by-one

T. A. Fulton

CURRENT in electrical devices is most often thought of as if it were the flow of a continuous, charged fluid. It is usually safe to ignore the actual discrete nature and charge ($-e$) of the individual electrons in this way because large numbers tend to be involved. Moreover, they screen each other, so that it is mostly just the average behaviour that is apparent. Recently, however, some small circuits cooled below 1 K have been devised in which current flow goes to the opposite extreme. In these 'single-electron' devices the electrons instead move one at a time in stepwise fashion, with the charge e playing a controlling role. One much sought result is to make this flow of electrons occur in lock-step with an applied radiofrequency signal, one electron per period, forming an ideal current source. A group from Chalmers and Moscow State Universities has already observed the onset of such behaviour¹, and now a team from Delft and Saclay has developed a circuit², called the single-electron turnstile, in which the synchronization is nearly complete, to about 1 part per 10^3 . Provided that this can be extended to 1 part per 10^7 or so, such a device could become the standard of current, much as the Josephson effect is the modern standard of voltage.

Single-electron circuits employ tunnel junctions of the sort used in Josephson work. These thin-film structures consist of an ultra-thin (1–3 nm) insulating layer sandwiched between two metal electrodes. At random times electrons will pass from one electrode to the other by quantum-mechanical tunnelling through the insulator. If a bias voltage V is applied between the electrodes, these

discrete, random events add up to a net current I through the junction, with a kinetic energy increment eV given to each tunnelling electron.

The essence of a single-electron device is that the current is required to pass, via tunnel junctions, through a region having low capacitance, C , to the rest of the circuit. One such configuration, of which the turnstile is an elaboration, has two closely adjacent junctions in series, with their common electrode forming the required region of low-capacitance (Fig. 1a). As current flows, tunnelling electrons come and go, and this region is repeatedly charged and discharged by an amount e . Each incoming electron must be able to supply an electrostatic energy of $e^2/2C$ to be allowed to enter. With junction dimensions at around $0.1 \mu\text{m}$ one can obtain $C < 1 \text{ fF}$ (femtofarad) which makes $e^2/2C$ equivalent to thermal energies at around 1 K. At temperatures well below 1 K and with bias $V \ll e/C$ there is too little energy available to overcome this barrier and the tunnelling rate is greatly reduced. This effect, now known as the Coulomb blockade³, was first observed many years ago in granular materials⁴.

The blockade can be lifted by lowering the electrostatic potential of the low-capacitance region using capacitive coupling to a nearby gate electrode. In this case an otherwise blocked electron borrows part of the energy needed to surmount the barrier from the gate voltage supply. This transistor-like control of the current by the gate was exploited by the Delft-Saclay group. Their turnstile device (Fig. 1b) had four adjacent aluminium junctions in series (total $R \sim 1 \text{ M}\Omega$) interconnecting three such similar low-capacitance regions ($C \approx 0.5 \text{ fF}$), and was operated at about 0.03 K. With this design they achieved a sort of one-electron shift-register by applying a low static bias across the four junctions from left to right plus a radiofrequency (f) gate voltage V_G coupled to the central low-capacitance region. The electrostatic energies work out such that when the gate voltage is maximum, one electron (only) can transfer to the central region through the two left-most junctions, but not farther. As the gate voltage decreases, it becomes possible for an electron to leave the centre and escape to the right. The two conditions do not overlap, so only one electron is transferred in each cycle.

In practice all this seems to have worked well. The current-voltage characteristics with $V_G = 0$ show a Coulomb blockade region of much enhanced resistance (greater than $1 \text{ G}\Omega$) at low bias, less than 0.2 mV. With a radiofrequency gate

voltage running at a few megahertz, the I - V curve in this region develops a nice constant current step at $I = ef$, about 0.16 pA MHz^{-1} , with a shape closely matching simulations based on a semi-classical model (Fig. 2). The precision with which I/f could be measured for $4 \text{ MHz} < f < 30 \text{ MHz}$ was limited by the difficulty in measuring the currents, a few picoamps, to within more than a few femtoamps. To give some idea of what more is needed before a standard of current can be achieved, the authors point out that for a precision of 10^8 , the frequency

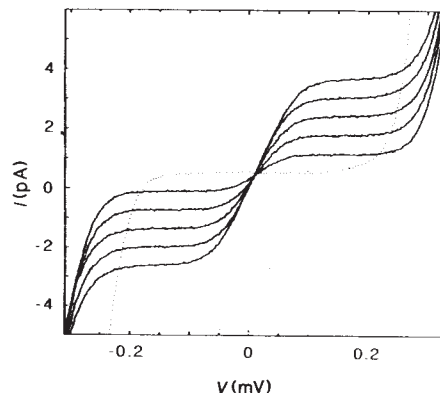


FIG. 2 Current-voltage characteristics of the new single-electron turnstile (from ref. 2). Dotted curve, no radiofrequency voltage applied. Solid curves, with applied radiofrequency voltage: current plateaus are seen at $I = ef$, where f is the applied frequency (4, 8, 12, 16 and 20 MHz) and e is the electron charge.

should be less than $10^{-3}/RC$, so that sufficient time is allowed in each cycle to be confident that the random tunnelling events do take place. This probably means that the frequency should be less than 100 MHz.

At the same time the number of electrons that leak through by some other path than through the turnstile mechanism must be less than $10^{-8}f$, or less than one per second. Within the semiclassical model, the only leakage is by thermally excited electrons passing over the Coulomb barrier. The authors calculate that a temperature of 15 mK would suffice to suppress these for the present device. As they note, however, it is uncertain whether a fuller quantum treatment will preserve this favourable prognosis. The Coulomb barrier itself has some spread in energy owing to its finite lifetime and the uncertainty principle, and this may cause an unavoidable leakage current. We shall have to wait and see. □

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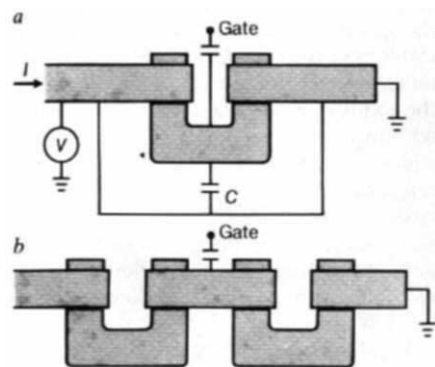


FIG. 1 a, Schematic of a one-electron device, comprising overlapping two Josephson junctions, metal electrodes separated by a thin layer of insulating material, with the central electrode connected to an external gate through a small capacitance (C). b, New turnstile device, an elaboration of the standard one-electron device. ($\perp$, Electrical earth.)

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Pieces of the folding puzzle

Robert L. Baldwin

AFTER many years of scepticism, it need no longer be doubted that proteins go through a series of identifiable intermediate stages as they fold up to assume their native conformations. In their latest work, reported on pages 440 and 488 of this issue^{1,2}, Fersht and co-workers show that the folding process in the enzyme barnase follows one preferred pathway with at least one distinct intermediate state: the ghost of the 'jigsaw-puzzle' model³, which postulates a random collection of folding intermediates, has now been laid to rest.

This new work is made possible by techniques for following the development of structure in a protein as it folds; questions can now be framed about how folding intermediates are stabilized and about the rate-limiting step in folding.

The first of the new methods^{2,4,5} essentially assays the state of individual peptide NH groups at different stages of the reaction. At various times after folding has been initiated, a protein with deuterated amide groups is pulse-labelled with hydrogen. Deuterons on the amide groups exposed to solvent at the time of labelling exchange for protons that can be detected when folding is completed by two-dimensional NMR analysis; as the amide groups become involved in structure they are protected from exchange.

Fersht and colleagues use their results to support deductions about the transition state and an intermediate in the folding of barnase that are based on the rates at which mutant barnase molecules fold and unfold, and on Fersht's general scheme for analysing the nature of protein folding interactions. They find that the secondary structure of barnase is formed early on in the folding process, in agreement with the 'framework' model⁶, which predicts that after an initial formation of secondary structure, pieces of the protein that have already folded form a framework on which the final folding process — fitting together the remaining side chains — can take place.

Similar results have been found for ribonuclease A (ref. 4) and cytochrome *c* (ref. 5). In the contrasting 'subdomain' model^{7,8}, the pieces of protein that dock to form the finished structure are already highly folded, with side chains in place and secondary structures already stabilized by tertiary interactions.

The barnase results suggest that the docking of 'preformed elements' late in folding is the rate-determining step in the folding process. Speculation is growing that the rate-limiting process involves stripping water from hydrophobic side chains as they become buried in the

protein's core. Mutational studies of the transition state and intermediate are found to be particularly useful in analysing tertiary interactions during folding¹. The barnase results suggest that most tertiary interactions are either fully formed or absent in the transition and intermediate state. Although a formal kinetic proof has not been given that the intermediates detected by exchange studies lie on the direct folding pathway, they seem to contain native-like structures consistent with hierarchical models of folding. Native-like structure is also observed in peptide models^{7,8} of early folding intermediaries of trypsin inhibitor stabilized by disulphide bond formation. Because the latter are stable intermediates, their structures have been analysed by standard NMR methods. The pulse-labelling results for barnase demonstrate that its folding intermediates lie chiefly on one preferred pathway and not on multiple pathways¹.

But just as structural evidence supporting the framework model has begun to appear, protein chemists have become intrigued with molten globule intermediates^{9,10}. These are stable intermediates that defy the two-state rule, according to which the folding reactions of single-domain proteins between the unfolded and the native states are completely co-operative, and stable intermediates (as opposed to kinetic intermediates) cannot be detected. Molten globule intermediates are compact and have substantial secondary structure but few if any fixed tertiary interactions; their hydrophobic side chains are not buried as in native proteins, but instead are exposed to water. They are found only in rare instances^{9,10}, typically at acid pH (as in the well studied example of α -lactalbumin) or when the native protein has marginal stability. Molten globule intermediates have recently been obtained for more proteins by careful adjustment of the salt concentration and choice of anion, after acid unfolding¹¹. In contrast to the conceptually based framework and subdomain models of folding discussed above, the molten globule model is based simply on the observed properties of stable molten globule intermediates and on the premise^{9,10} that similar forms lie on the kinetic pathways of protein folding. Fersht and co-workers find that the hydrophobic core of barnase is only partly organized in the transition state and intermediate¹, consistent with a molten globule model.

There are several good reasons for the current curiosity about molten globule intermediates. First, they violate the two-state rule: what happens to the co-operativity of folding? Second, hydro-

phobic side chains, buried in the native protein, are still exposed to water in molten globule intermediates^{9,10}. If the burial of hydrophobic side chains is chiefly responsible for stabilizing the structures of native proteins, what stabilizes molten globule intermediates? Can hydrophobic side chains in contact with water nevertheless interact to stabilize folding intermediates? Benzene forms dimers in water, although weakly¹², and this effect provides a basis¹³ for addressing the question.

Another reason for interest is the question of why the compact conformations of molten globule intermediates should be linked to the appearance of secondary structures, when there are few, if any, tertiary interactions^{9,10} to stabilize the secondary structures. Unfolded staphylococcal nuclease assumes a compact conformation at low concentrations of denaturant¹⁴ and presents a similar puzzle. It is likely that, in both cases, the compact conformation is produced by some kind of hydrophobic collapse. But why should this cause the appearance of secondary structures, which are held together by hydrogen bonds? Chan and Dill¹⁵ suggest an answer to this question. They generate random compact conformations by folding on a lattice model: secondary structures turn out to constitute a large fraction of the resulting compact conformations. Consequently, occurrence of a hydrophobic collapse, if it happens, could be sufficient explanation for the appearance of secondary structures. Chan and Dill ask why secondary structures are so prevalent in native proteins but their result applies also to other compact conformations, such as molten globule intermediates.

Another piece of the puzzle has been added by Baum and co-workers¹⁶, who use NMR to determine which amide protons are protected in the molten globule intermediate of guinea-pig α -lactalbumin. They find that two segments that form α helices

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in the native protein have protected protons in the molten globule intermediate (J. Baum, personal communication).

But the mystery of the molten globule intermediates now gets darker. An important motivation for studying protein folding pathways is to find the principles that relate amino-acid sequence to three-dimensional structure. Protein chemists have become used to thinking that the chief structure-determining principle is based on close packing of hydrophobic side chains in the protein core¹⁷, but the hydrophobic side chains are still exposed to water in the molten globule. What, then, determines that the helices that form in the molten globule of α -lactalbumin are similar to those in the native protein?

As if these conundrums were not enough, Pitsyn and co-workers¹⁸ have

now added another. They propose first that molten globule intermediates occur universally on the kinetic pathway of protein folding and then present evidence suggesting that the molten globule is formed only after secondary structure appears, which contradicts the above suggestion that secondary structures are formed because the molten globule has a compact conformation. If a hydrophobic collapse is not needed to produce secondary structures in folding intermediates, then what does stabilize them? The trail seems to be leading back towards the framework model. It is a safe bet that new developments will come rapidly. □

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GLACIAL CLIMATE

Ice masses on the move

Donald G. Sutherland

DURING the last glacial cycle (approximately 120,000 to 10,000 years ago) the maximum expansion of ice sheets is generally presumed to have occurred at around 18,000 years before present (BP). Glacial advances pre-dating this time are not well documented, apparently because of the destruction of evidence by that last ice advance as it over-rode the deposits of the earlier glaciations. The work of Seret *et al.*, reported on page 453 of this issue¹, is therefore of particular interest. They apparently demonstrate that at least one phase of glaciation of the Vosges mountains (at approximately 48° N) during the last glacial cycle was considerably more extensive than that which occurred at the last glacial maximum. An extensive end moraine system that marks the maximum extent of the ice is reported, and dating control on the ice advance is derived from long sequences of terrestrial and lake sediments preserved in basins outside and inside the ice limits.

The interglacial/glacial/interglacial cycle of the past 125,000 years is important in elucidating the mechanisms controlling the Earth's climate. As it is the most recent such cycle, the relatively well preserved and geographically widespread deposits dating from it allow detailed reconstruction of particular events. The period offers examples of large, rapid changes in climate as at the end of the last cycle when global temperatures may have increased by around 5 °C over a few centuries (or less). It is also possible to recreate the palaeogeography and palaeoclimate of certain periods characterized by climates markedly different from the present climate and to use these reconstructions to test computer models of global circulation.

The distribution of glacial ice is of great importance for palaeoclimatic modelling. Ice has a crucial effect in reflecting solar radiation: the largest ice sheets were of sufficient size to modify the general circulation. And individual ice masses also had direct effects on local climate, modifying, for example, precipitation patterns in their immediate vicinity. But although the general trends in climate during the last glacial cycle have been indicated by analyses of deep-sea sediments or ice cores from the Greenland and Antarctic ice sheets, these continuous records are unable, in other than a rather general way, to indicate the geographical expression of the environmental changes they record. Thus, for example, although it may be possible to infer that a certain volume of ice existed during a certain period, little can be deduced as to where that ice had accumulated. Similarly, rapid changes in temperature, whether positive or negative, can be inferred from the continuous records, but this tells little of the environmental response to these changes. It is necessary, therefore, to turn to the continental record to discover the evidence relevant for palaeogeographical reconstructions.

In their study¹, Seret *et al.* describe a series of terminal moraines formed by an ice mass emanating from the Vosges mountains in France. They establish that these moraines are more than approximately 30,000 years old, from the sequence of sediments in the Cusenier basin which lies within the outermost limits of the ice advance. In addition, they use the long sequence of terrestrial environmental change interpreted from the sediments preserved in the neighbouring Grand Pile peat bog to suggest that the

most probable period for the glaciation was between 30,000 and 50,000 BP. They do not rule out the possibility of earlier glaciation, however, nor, as the moraines marking the limit of the ice advance are multiple, the possibility that there was more than one period of glaciation.

Their favoured timing of the glaciation falls within oxygen-isotope stage 3, a period some would consider relatively mild, although one in which the deep-sea record indicates there to have been substantial global ice volume. As noted by Seret *et al.*, the southern margin of the Laurentide ice sheet was apparently well advanced during this period² and comparison may also be made with the western Norwegian sequence³ where glaciation of the coast during the Skjonnhelten Stadial (between approximately 60,000 and 30,000 BP) has been inferred.

The dynamics of an ice mass is a question of balance between accumulation and ablation, the former generally being aided by increased solid precipitation and the latter largely being controlled by temperature. As the precise balance between these two major variables for a particular area may differ somewhat from one cold period to another then the dimensions of any consequent glacial build-up need not be similar in any two such periods. Seret *et al.* use evidence from minerals and pollen from the Grand Pile bog to address this problem and suggest that the principal local control on ice expansion and contraction in the Vosges was precipitation rather than temperature.

Thus they envisage the ice building up during a period of cool but not extremely cold climate, a period characterized by high snowfall and with the ice in retreat as much in response to a decrease in snowfall as to an increase in temperature. Although they do not directly state it, there is the further possible implication that a different combination of precipitation and temperature occurred during the build-up of the ice immediately before about 18,000 BP. This could explain the smaller ice mass that developed in the Vosges at the later time.

This work of Seret *et al.* highlights a current lack of knowledge about major ice masses during the last glacial cycle. With the broad temperature trends during this period already relatively well known, more work along these lines could yield a better idea of the contemporaneous changes in precipitation patterns. With that, we would have a near-complete picture of the glacial climate. □

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African dawn for primates

Philip D. Gingerich

THE fossil record of primates is one of the most intensively studied of all major mammalian groups. But until recently, fossils of the most ancient true primates were absent from Africa, where primates are a diverse faunal component today. This gap has now been filled with the discovery of the first true primate from the Palaeocene of Africa, by Sigé *et al.* at the University of Montpellier in France¹.

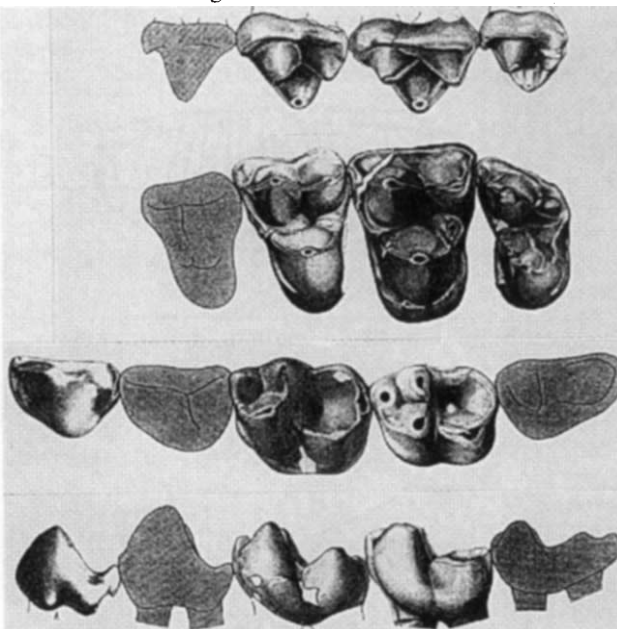
This new find, *Altiallasius koulchii* Sigé, comes from late Palaeocene sediments at Adrar Mgorn, in the eastern Quarzazate Basin of Morocco, at the foot of the High Atlas mountains, a locality discovered in 1977 (ref. 2). Fossils occur in a hard calcareous matrix deposited in a nearshore marine setting. These are fragile and extracted with difficulty in acid. Shark teeth indicate that the age is Thanetian (late Palaeocene, about 60 million years ago)³. Twenty-three mammalian species are represented in the Adrar Mgorn fauna^{4,5}, some by beautiful dentaries and maxillae, but most by isolated teeth. Small mammals predominate, especially insectivorous species. All are eutherians, including genera familiar in the Palaeocene of Europe or North America (*Aboletylestes*, *Cimolestes* and *Palaeoryctes*) showing that communication with northern continents was possible at times.

The new primate is represented by ten isolated cheek teeth (see figure). One dentary fragment of a juvenile preserves a single erupting molar, but no two teeth were found in association. The upper molars are trapezoidal at the base with a broadly basined trigon. The lower molar trigonids are low and talonids too are broadly basined. Tooth size indicates that *Altiallasius* was comparable in body size with the mouse lemur *Microcebus murinus* or Demidoff's galago *Galago demidovii*. In life, *Altiallasius koulchii* probably weighed no more than 50–100 grams.

Sigé and colleagues assign *Altiallasius* to the family Omomyidae and regard it as the oldest haplorhine and the oldest true primate. They note close resemblances with younger, possibly more derived forms such as *Omomys* and *Chumashius* from the Eocene of North America⁶ and *Kohatius* from the Eocene of southern Asia^{7,8}.

The single known premolar is unusual and does indeed suggest an affinity with *Kohatius*. Sigé and colleagues also compare *Altiallasius* favourably with the

contemporary plesiadapiforms *Berruvius* from the Palaeocene of Europe and *Micromomys* from the Palaeocene of North America, and to a lesser degree with the adapid *Donrussellia* from the early Eocene of Europe and the adapid or catarrhine *Oligopithecus* from the Oligocene of Africa. Bulbous cusps on lower molars remind me a little of *Cantius*. Sigé and colleagues do not discuss Asian



Left upper and lower cheek teeth of *Altiallasius koulchii* Sigé, a late Palaeocene primate from Adrar Mgorn in Morocco. Upper molars (top) are shown in lateral and occlusal view. Lower molars (bottom) are shown in occlusal and lateral view. The largest upper molar is the holotype, and it measures 1.75 mm in length and 2.45 mm in width. No teeth were found in association, so the position of each in the tooth row is necessary conjectural. Drawings by Ariane Beauw and Christian Pondeville.

Altanius, but *Altiallasius*, like *Altanius*, is sufficiently primitive that it does not fit clearly into any single familial grouping⁹. Sigé and colleagues are probably right that *Altiallasius* is an omomyid and the oldest true primate, but isolated teeth are difficult to interpret and more complete specimens with anterior teeth will be required to remove some lingering doubt.

The African origin of primates is an old idea¹⁰, but fossil evidence to support it has emerged only in recent years. The discovery in 1975 of *Azibius* in Eocene sediments in Algeria¹¹ — the most ancient primate then known from Africa — helped convince me that primates originated in Africa¹², although others preferred a centre of origin in central^{13,14} or southern¹⁵ Asia. Since then, further discoveries in Algeria¹⁶ and Egypt¹⁷ have added weight to the idea of an African origin. Now we have *Altiallasius* from the Palaeocene of Morocco. Another new form, from the Eocene of Tunisia, is under study at Montpellier by Hartenberger and Godinot. Taken together, these specimens provide strong support for an African origin of primates. Diversification in Africa in the late Palaeocene followed by northward dispersal when climates warmed globally across the Palaeocene/Eocene boundary¹⁸ may explain why true primates are not found on northern continents until the early Eocene.

Sigé and colleagues conclude by ranking *Altiallasius* as the sister group of Anthropoidea (Simiiformes), and suggest that it indicates, first, that anthropoid primates differentiated during the Palaeocene, and second, that platyrrhine anthropoids (New World monkeys) rafted the Atlantic Ocean in the Palaeocene when Africa and South America were closer together. Some doubt remains that *Altiallasius* is a true primate (after all, its teeth compare well with some plesiadapiforms), and it is certainly not an anthropoid. If *Altiallasius* is not an anthropoid, then it does not prove anthropoids existed, let alone differentiated, in the Palaeocene; and crossing the South Atlantic in the Palaeocene would have been a big stretch when *Altiallasius* seemingly could not cross the Tethys Ocean to Europe. □

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The X, Y, Z of head development

P. W. Ingham

MOLECULES that can trigger a series of biochemical switches are much sought after by developmental biologists, so the cloning and characterization of the *Drosophila* gene *bicoid* has generated considerable interest and excitement over the past two years. Yet despite being hailed as a paradigm *par excellence* of such a biological morphogen¹, a good deal of mystery has continued to surround the workings of the *bicoid*-encoded homeodomain protein in the early embryo. Although displaying the proverbial graded distribution², the capacity of the *bicoid* protein to evoke a series of threshold responses has been difficult to prove other than in principle, because its targets have for the most part remained obscure. Several recent studies³⁻⁵, two of which are reported on pages 482 and 485 of this issue^{4,5}, have now identified such target genes, opening the way to a detailed analysis of the molecular basis of threshold responses.

A chief obstacle to the full understanding of *bicoid* (*bcd*) function has been the dearth of information about genes controlling development of the head, the part of the embryo requiring the highest levels of *bcd* activity. Posterior to the cephalic fold — a deep invagination of cells which separates the presumptive head from the rest of the embryo during early gastrulation — the appearance of primordial segments can be readily visualized through the striped expression patterns of pair-rule genes such as *fushi tarazu* (see figure). These genes are pivotal in the early development of the embryo, effecting the transition from aperiodic regional differences, generated by the selective expression of gap genes (such as *hunchback*, *Krüppel* and *knirps*), to the repeating patterns that underlie its segmental organization⁶. Anterior to the cephalic fold, by contrast, pair-rule genes seem to have little if any function and their expression is correspondingly sporadic (*hairy*, *runt*, *paired*) or, in the cases of *even-skipped* and *fushi tarazu*, nonexistent.

Whereas pair-rule genes have been useful in monitoring the consequences of altered levels of *bcd* activity⁷, the effects of such alterations on their expression are in fact somewhat limited: in embryos lacking all *bcd* activity, the two anterior-most stripes disappear, but decreasing or increasing levels of *bcd* protein simply decrease or increase the void in pair-rule expression at the anterior end of the embryo. What has remained mysterious is the identity of the genes that fill this void and specify the organization of the head.

In the absence of putative head-specific-

ing genes, the search for *bcd* targets was focused initially on the gap gene *hunchback* (*hb*). In common with other gap genes, *hb* controls the generation of particular pair-rule stripes, specifically the two most anterior which disappear in *bcd* mutant embryos. Zygotic expression of *hb* extends, like *bcd* protein, from the anterior pole to the middle of the embryo (100–50 per cent egg length (EL)) and is absolutely dependent upon *bcd* activity⁸. In several papers published last year, Driever, Nüsslein-Volhard and col-

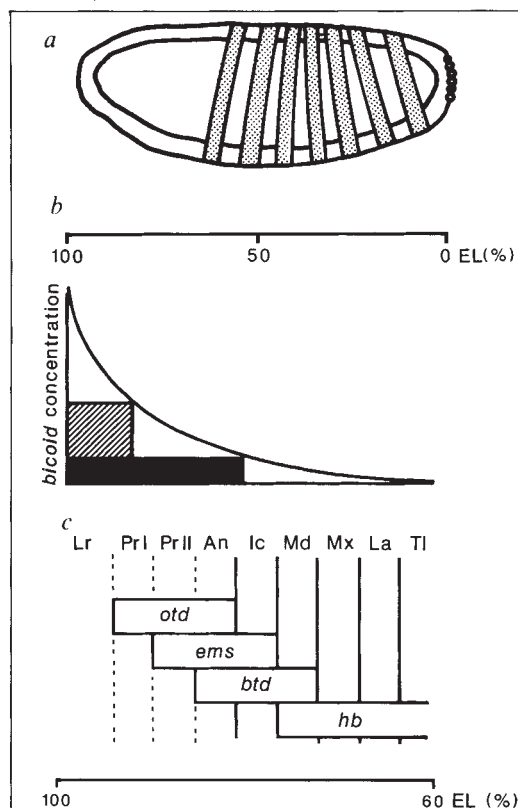
regulatory sequences can elicit different responses to the *bcd* gradient. DNA footprinting analysis revealed two distinct types of binding sites upstream of the *hb* promoter, one of which binds *bcd* protein with high, the other with low affinity. Whereas the high-affinity sites alone are sufficient to drive expression of a reporter gene throughout most of the *bcd* concentration range in a manner similar to the endogenous *hb* gene, the apparently redundant lower affinity sites, can, when isolated, direct expression in a much narrower domain, restricted to the anteriormost quarter of the embryo where *bcd* levels are highest. Because the absence of *bcd* results in more defects in anterior development than those caused

by *hb* mutations, it had already been recognized that additional genes should be under the control of *bcd*: but here was the first indication of where such a gene — termed *gene X* by Nüsslein-Volhard and colleagues — might be expressed and how such an expression domain might be defined (see figure).

In December 1989, Dalton, Chadwick and McGinnis³ described the cloning of a possible candidate for *gene X*, the *empty spiracles* (*ems*) gene. At first sight, this seems an unlikely match because the name *empty spiracles* refers to a defect in the tracheal system originating at the posterior end of the embryo. But more detailed analysis of *ems* mutants suggested an additional role for the gene in the differentiation of specific head structures, an inference made more compelling by analysis of the spatial regulation of *ems* expression. The gene was cloned by virtue of the fact that it encodes a protein with a homeodomain, distantly related to that encoded by *even-skipped*. Using antibodies specific for the *ems* protein, Dalton *et al.* showed that its expression is initiated in the blastoderm embryo in a wedge-shaped band of cells between 70 and 88 per cent EL, overlapping the *gene X* domain. Moreover, the position of this band depends critically on *bcd* activity, shifting

respectively anteriorly or posteriorly with decreasing or increasing doses of the gene.

An even closer fit to the artefactual *gene X* expression pattern is provided by the distribution of transcripts of the *orthodenticle* (*otd*) gene, described by Finkelstein and Perrimon on page 485 of this issue. Like *ems*, *otd* encodes a homeodomain protein¹³, in this case exhibiting a similarity with the homeodomain of the *bcd* protein itself. Expression of *otd* is also restricted initially to the anterior part of



a, Expression of *fushi tarazu* in the blastoderm embryo is restricted to the prospective 'trunk' region of the embryo and absent from the prospective head (left) where *bcd* levels are highest (see b). b, Graded distribution of *bcd* protein and the expression domains of *hunchback* (shaded) and the hypothetical *gene X* (hatched) (adapted from ref. 2). c, Overlapping functional domains of *otd*, *ems*, *btd* and *hb* in the head segments as deduced from mutant analysis (adapted from ref. 5).

leagues⁹⁻¹¹, and Macdonald, Struhl and Struhl¹², demonstrated that the *bcd* protein acts directly on regulatory sequences upstream of the *hb* promoter to activate *hb* expression. Although this finding was gratifying inasmuch as it demonstrated the transcription factor-like character of the *bcd* protein, it shed little further light on the significance of the graded distribution of the protein.

But what was more exciting was the discovery that different parts of the

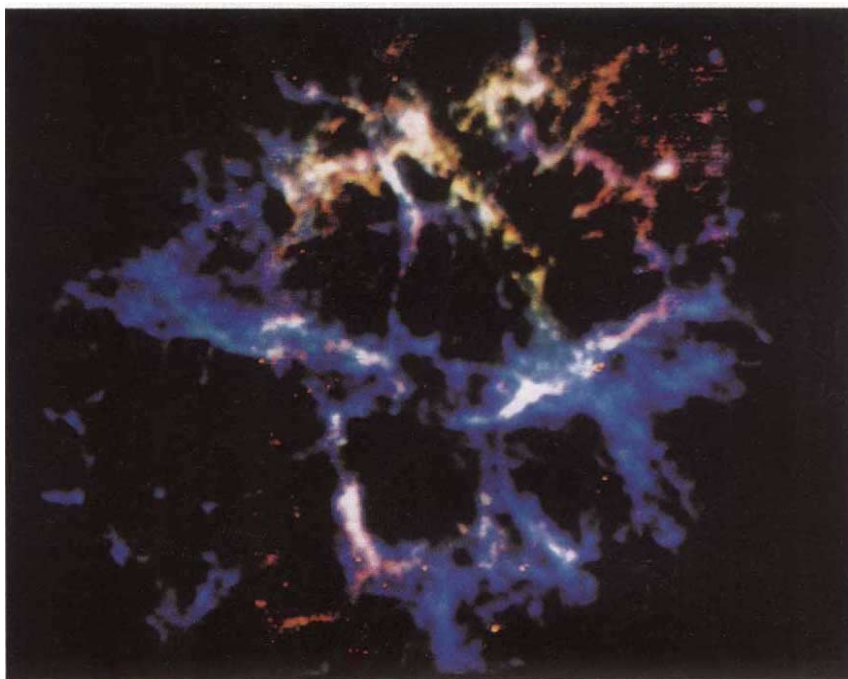
the embryo, in a uniform band of cells normally located between 70 and 90 per cent EL, the posterior limit of which is sensitive to decreased or increased *bcd* dosage. A clear implication of these studies is that both *otd* and *ems* are activated in spatially restricted domains in the blastoderm embryo in direct response to levels of *bcd* protein. Whether this response is mediated via the same low-affinity binding sequences as those located upstream of *hb* will doubtless be revealed soon.

The *otd* mutation was originally characterized as showing defective head involution¹⁴. This rather gross phenotypic description reflects the difficulties in analysing the final intricate pattern of the head segments in the mature larva — largely due to the complex topological rearrangements occurring in the head during embryogenesis — which have bedevilled the study of this part of the animal. To circumvent this problem, Finkelstein and Perrimon used the products of the *wingless* and *engrailed* genes as molecular markers for head segments in early embryos, and they were thus able to identify unequivocally those segments missing in *otd* mutants.

Using a similar approach, Cohen and Jürgens have extended this analysis not only to *empty spiracles* but also to mutations of *buttonhead* (*btd*), a third gene that affects head development. Their findings reveal overlapping requirements for the activities of all three genes in the specification of each of the head segments anterior to the cephalic furrow (see figure). Because the functional domains of each gene appear to be out of register by one segment, Cohen and Jürgens propose that they act in a combinatorial fashion to specify the boundaries of each of the head segments, probably by directly regulating the expression of *wingless* and *engrailed*. They may also determine the identities of each segment by regulating the expression of head-specific homeotic genes such as *labial* and *Deformed*^{15,16}. In this way, these head-specific 'gap' genes would subsume the functions normally executed by gap and pair-rule genes in the more posterior segments of the embryo.

Although this interpretation is persuasive, the precise relationship between function and expression of the three genes remains unclear: although *ems* is evidently required more posteriorly than *otd*, for

Fine filaments of the Crab Nebula



ILLUMINATED by a powerful pulsar at its heart, the Crab Nebula has continued to fascinate astronomers since it was discovered by Lord Rosse in 1844. What we see are the gaseous ejecta from a massive supernova that Chinese observers could see even during daytime 940 years ago. The nebula is now 4 light years across and is expanding at a rate of 1,400 km s⁻¹. The filaments traced out by the ejecta are the result of turbulence and shockwaves. In making this new three-colour overlay figure (*Astrophys. J.* 357, 539–547; 1990), Jeff Hester and colleagues intend to understand better the chemical composition of the nebula: red indicates iron (recorded using new infrared imaging), green, hydrogen and nitrogen, and blue, oxygen. In fact the varying colouring of the filaments indicates on the whole not varying composition, but rather differing conditions, although an excess of hydrogen swept up from the interstellar medium is apparent above the middle of the figure. One aim of the study is to tackle the iron–nickel problem in astronomy. The best measure we have of the cosmic abundances of the elements comes from studies of the composition of the Sun and meteorites formed billions of years ago from gas clouds comprising hydrogen and helium and enriched with heavier elements by supernova remnants similar to the Crab Nebula. Yet these measurements indicate that there is 10 times more iron than nickel, whereas their apparent abundances in supernova remnants and in star-forming clouds are equal. The authors conclude in the new study that the problem must arise through difficulties in the interpretation of the atomic data, and does not reflect a real variation in astrophysical abundances. □

instance, the domains of expression of both genes appear to be essentially co-extensive. But if shown to be correct, there is an intriguing irony in all of this. Recent studies of the hindbrain in chicks and mice^{17–19} suggest a close analogy between metamerism in invertebrates and an underlying segmental organization of

the vertebrate nervous system. This analogy has been much strengthened by the pair-rule-like expression of the mouse *Krox 20* gene — yet it would now seem that the comparable region of the fly embryo may be segmented by a quite distinct mechanism, independent of pair-rule genes. Had the analysis of *Drosophila* development begun with the *bcd* system, the specification of head segments by overlapping domains of homeobox proteins might well have been established as the archetypal gene hierarchy: what then might have been made of zinc-finger protein stripes in alternate rhombomeres? □

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The Hyperion hypothesis

William B. McKinnon

ORBITING just beyond Titan in the Saturn system is Hyperion, a $350 \times 240 \times 200$ km icy fragment of a world (Fig. 1). Its curious shape strongly suggests that it is the remnant of a catastrophic breakup. Indeed, it is generally thought that during the first few 100 million years of the evolution of the Solar System, all of the inner satellites of Saturn, Uranus and Neptune were repeatedly shattered by collisions and then reassembled. What sets Hyperion apart from many of these

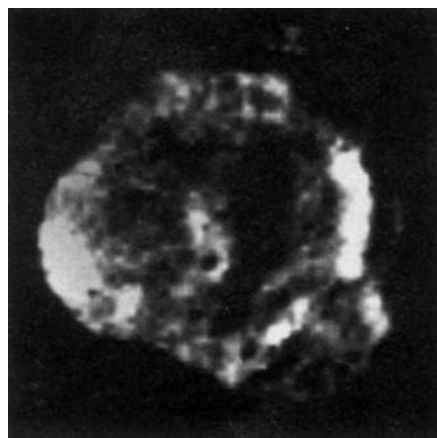


FIG. 1 Saturn's Hyperion: remnant of a proto-satellite and father to a crater population?

ostensibly rebuilt moons is its shape — apparently, its fragments were not able to re-accrete into the relatively spherical form usually expected for satellites of its size. This suggests that Hyperion is itself only a fragment of a previously more massive satellite and that the other pieces (or most of them) did not return home. Exactly where Hyperion's fragments might have gone, or more specifically, which other satellites might have captured them is the subject of a recent paper by Farinella *et al.*¹

Actually, the matters addressed in this paper are broader than that. The central issue is the origin of crater populations, and the overall subject is nothing less than the bombardment history of the Solar System. This history is the result of the complex interaction of impactor sources and dynamic delivery mechanisms, and their evolution over time. Most impacts, however, occurred very early in the Solar System's lifetime, and are attributable to remnant populations from the accretionary processes that originally built the planets and their satellites. Thus, the heavily cratered surfaces record the last stages of accretion of the Solar System. If properly deciphered, this record would help us to understand the construction of our Solar System — and others, if they exist.

Hyperion's fragments enter into this

through the interpretation of crater counts, or size-frequency distribution curves. Some researchers regard the heavily cratered terrains on most planetary and satellite surfaces as having essentially the same distributions². Others interpret the differences in form between distributions to be significant. Conclusions concerning the impactor populations are just as varied. Some hold that nearly all cratering in the Solar System was been caused by comets, both ancient and modern³. Others advocate that the different forms of crater distribution curves for different bodies must be explained by physically different impactor populations. In particular, Strom, one of the co-authors of the new paper¹, argues that the jovian, saturnian and uranian satellites have been bombarded by different populations, and thus rejects the idea that comets, the plausible generic impactors of the outer Solar System, were the cause. He holds that each satellite system was bombarded by its own, unique impactor population that originated from orbits around the parent planet¹.

Saturn's icy satellites, in addition, have been held to record two distinct populations. One population (number I) is found on the ancient, heavily cratered regions of Tethys, Dione and Rhea, and also dominates inner Mimas; the other (number II) is also found on most heavily cratered surfaces, and is superposed on the younger, resurfaced areas of Enceladus, Tethys and Dione. The younger population II has a relative excess of small craters less than about 20 km across, and is thus recognized on heavily cratered Rhea, for example, by a bump in the crater size-frequency distribution (Fig. 2). The original hypothesis for the origin of population II craters was secondary cratering: the debris scattered by population I impacts would create subsidiary impressions⁴. This idea was expanded to include the distributed effects of the collisional breakup of small satellites⁵, and Strom focused this hypothesis on Hyperion⁷. More importantly, recogni-

tion of a system-wide breakup event or events offers the possibility of establishing internal clocks for the geological evolution of Saturn's satellites.

Farinella *et al.*¹ track the fate of Hyperion's fragments by realizing that the agent of their misfortune is its neighbour, the (relatively) massive moon, Titan. Had Titan not been where it is, much of the debris from an impact on Hyperion would have rapidly re-accreted onto the original moon, albeit in a re-ordered form. But as it is, the orbital periods of Hyperion and Titan have an exact mathematical relation of 4:3 (with Hyperion's the longer), and over repeated orbits this resonance can cause the orbits of fragments to evolve chaotically, with drastic consequences. Even those fragments with velocities that would otherwise have left them in a belt relatively close to Hyperion would find themselves pulled into orbits that crossed Titan's⁷. This was the likely cause of Hyperion's inability to re-accrete.

Farinella *et al.* consider Titan's

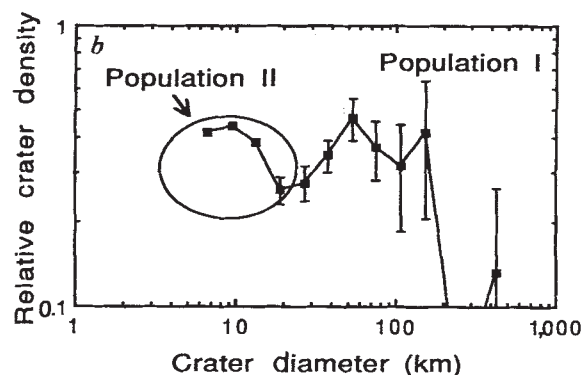
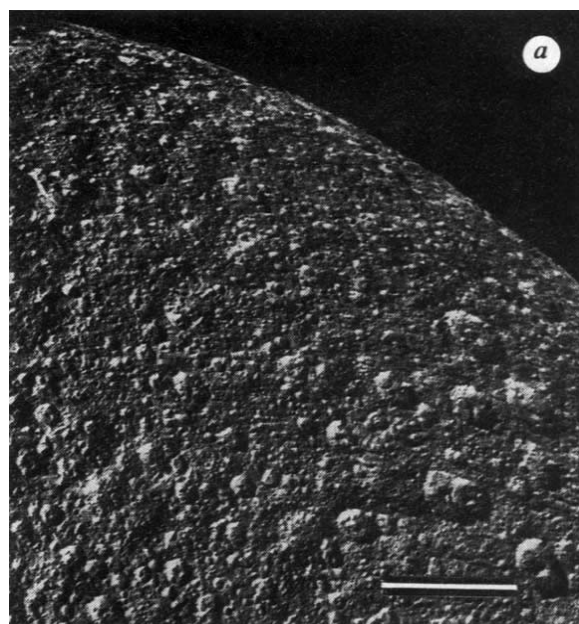


FIG. 2 a, A portion of the north polar region of Rhea, as seen by Voyager 2. Population II manifests as the peppering of small craters (scale bar, 100 km). b, Statistically, population II shows up as a bump in the relative incremental size-frequency distribution⁴, whereby the areal density of craters per diameter (D) bin is normalized (divided) by D^{-3} .

influence on two classes of Hyperion's fragments, those that chaotically evolved so that they barely crossed Titan's orbit and those that were placed directly into Titan-crossing orbits. This latter class arises from the uncertainty in the velocity distribution of ejecta from breakup events. Outcomes are determined using Öpik's statistical theory of orbital diffusion⁸. The robust inferences are first that Titan swept up the lion's share of any fragment swarm; and second that of those fragments that did not rain down on Titan (up to around 10^{-1} of the total), Rhea, the next satellite in, got the majority. The amount of disrupted mass reaching each successive inner satellite decreases sharply. The amount of material reaching inner Mimas, which is dominated by population II craters, was negligible.

What this means is that the Hyperion hypothesis fails: Hyperion was not the source of system-wide planetocentric cratering events. Furthermore, Titan, by virtue of its relatively great gravitational cross-section, acts as a dynamic shield in the Saturn system. Any planetocentric impactor population lurking beyond Iapetus, Saturn's outer moon, which is the only plausible place to store long-lived planetocentric impactors⁹, would have had relatively little effect on the satellite system inside Rhea. A non-negligible fraction of Rhea's population II craters may have been caused by Hyperion's fragments, however. To account for all of Rhea's population II craters requires, for plausible mass distributions of the fragments, a total ejected mass of the order of Hyperion's present mass, if indeed about 1 in 10^4 fragments hit Rhea¹. Because the sphere that just encloses Hyperion's present figure has three times its present volume, this mass requirement is not implausible.

If Hyperion (or some hypothetical trans-Iapetus population) was not the cause of the population II craters on Mimas, Enceladus, Tethys and Dione, how were these craters formed? It would seem that local sources are still the most likely, that is, ejecta from giant craters or basins, such as exist on Mimas, Tethys and Rhea, and debris from the collisional breakup of small satellites such as the irregularly shaped Trojan companions to Dione and Tethys. That Rhea's popula-

tion II craters could be caused by ejecta from its large basin Tirawa is not far-fetched, because the efficiency of Rhea's accretion of Hyperion debris of around 10^{-1} is actually a rough upper limit that depends on favourable geometrical or dynamic circumstances. As to the ultimate origin of these large craters and small satellite breakups, they were most likely caused by heliocentric impactors, probably comets or comet-like bodies¹⁰.

Although the work of Farinella *et al.* elucidates only one aspect of the bom-

EVOLUTION

The language of the genes

N.H. Barton and J.S. Jones

GENETICS is a language, a set of instructions passed from one generation to the next. Genes and languages each possess a history and have changed as their carriers succeeded each other and moved over the world. The history of language — its evolution — can be traced from the similarities and differences of living tongues and their literary fossils. In the same way, shared genes can be used to trace ancient patterns of relatedness. Two new papers^{1,2} have uncovered some remarkable parallels between the geography of genes and of language which suggest that they have a common evolutionary history.

There are obvious analogies between linguistic and genetic evolution. Each depends on the origin of new coding elements by mutation, on random or directed change in their frequency, and on their spread by diffusion or migration. The idea of an evolutionary clock was first proposed in the nineteenth century by a linguist — appropriately enough, some might claim, Grimm of fairy-tale fame — who suggested that the differences between living languages could be used to trace their divergence. Both the phonetic and the molecular clock keep bad time if, as is true for certain proteins, bird songs and human speech patterns, some elements reproduce more effectively than others^{3,5}.

The geographical link between genes and language emerges from studies of many thousands of Europeans scored for cell-surface antigens and polymorphic enzymes. New statistical tests¹ show that Europeans with the same language are on average more genetically similar than are those speaking different tongues. Geographical trends in the frequencies of 63 alleles reveal 33 sharp boundaries which separate genetically distinct populations²: 26 of these coincide with differences in language and five are boundaries between dialects. Some populations — such as the Basques — are linguistically and genetically quite different from others.

In spite of these linguistic effects, most

bardment history of the Solar System, it illustrates the complexity of the problem very neatly. Much further work can be done, even for the Saturn system, using the full numerical armamentarium of celestial mechanics. As with much in chaotic dynamics, we should expect surprises, even startling ones. □

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genetic variation is due to proximity, and neighbouring populations cluster without much regard to linguistic relatedness⁶. The fit between genes and language is subtle and can be seen only after sophisticated statistical analysis. The general pattern is of localized random fluctuations, probably produced by a balance between random drift and the diffusion of genes from place to place. Only on the larger scale are past movements of whole populations manifest as boundaries between languages.

Mass Movement

Local diffusion and mass migration — whether expansion from within an existing range, or by invasion from outside — are extremes in the complex picture of human gene flow. From the Old Testament to *Mein Kampf* history has seen mass movement as the key to the peoples of the world. By contrast, population geneticists have concentrated on within-population processes such as genetic drift and the diffusion of genes. These may be enough to explain the local differentiation of tribal and agricultural peoples, but they cannot be the whole story. Because genes diffuse through settled human populations very slowly — with a standard deviation of perhaps 1 km in a year⁷ — it would take around 10,000 years for a new mutation to spread over 100 km. Mass movement must have helped to determine the population structure of modern humans. Concordance between unrelated characters usually indicates a meeting of differentiated populations, as opposed to differentiation *in situ*⁸. The European genetic boundaries and their coincidence with language probably reflect such secondary contacts.

The population genetics of bulk migration has scarcely been explored even though, over large scales, it must be more important than gradual diffusion. Phylogenies based on mitochondrial DNA in various creatures suggest that it is widespread⁹. In many species, plots of genetic against geographic distance show that

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widely separated populations are much more similar than expected by diffusion alone¹⁰. There is a similar pattern in the relationship between language and geography in Polynesia¹¹.

Trends in gene frequency from south-east to northwest Europe may reflect demic diffusion¹², a process intermediate between local diffusion and mass migration. As early farming communities expanded and spread west, they interbred with local hunter-gatherers. Genes in the east are typical of the newcomers, whereas those in the west retain an admixture from the original inhabitants. Most European languages — excluding Basque and a few others — belong to the Indo-European family which may itself have its roots in the origins of agriculture in the Fertile Crescent¹³.

Horizontal transmission

Unlike genes, language can spread horizontally. In some places, this has destroyed the relationship between genetic heritage and speech. In the Pacific, for example, there is a sharp genetic change near Fiji in spite of a smooth gradient in language¹⁴. Bird-song dialects rarely last for more than 20 generations, and are transmitted by learning, with no genetic differences between populations with distinct dialects¹⁵.

Shared patterns of human genes and language around the world suggest, however, that such horizontal transmission was not widespread in the sparse human populations of earlier times, as the parallels between the evolution of genes and language reach back into prehistory. A phylogeny of 42 human populations based on 120 gene markers fits well with a world tree of language superfamilies¹⁶. For example, there is an ancient genetic split between African and non-African populations which accords with the distinctiveness and diversity of sub-Saharan languages. The origin of language could even have been the catalyst for the spread of modern humans across the globe.

A language is a group of mutually intelligible dialects. This definition is close to that of a biological species: a group of actually or potentially interbreeding populations. Like species, languages are more-or-less closed evolutionary units which cannot easily be penetrated. As it is hard to measure gene flow or mutual intelligibility, taxonomists and linguists are forced to use morphological criteria when defining the units they work with.

A problem common to linguistics and biology is just why such morphological units exist: why should inherited information be divided into distinct clusters? Perhaps clustering is an inevitable consequence of evolution. Anything which — like species and languages — evolves by descent with modification automatically produces discrete taxa¹⁷. This may be the

only reason why asexual species can be defined. Mutual recognition, which is involved in sex and in social structure, binds local populations into tighter linguistic and genetic clusters than does common descent alone. The cohesive effect of shared signals is seen in the warning colours of mullerian mimics — in *Heliconius* butterflies, regions of hybridization between areas with contrasting signals are narrow, as hybrids are less fit than those giving an unambiguous warning message¹⁸. A similar mechanism could explain why the boundaries between dialects are so sharp: for example, the pronunciation of some vowels changes over a few kilometres between the English Midlands and East Anglia¹⁹.

What of the future? Increased mobility and the frequency-dependent disadvantage of rare languages mean that linguistic isolates are now endangered species. The diffusion of genes and languages will tend to smooth out barriers. For example, Low and High German are distinct dialects separated by a sharp linguistic boundary across northeastern Germany. In the Rhine delta, which has been settled for much longer, they have become blurred: separate components change over in different places¹⁹. It is still an open question whether the newly found concordant genetic boundaries that subdivide Europe are just the neutral relics of ancient movements, or whether they are actively maintained by linguistic barriers. Detailed studies of the genetics of frontier populations are needed before we can understand what shaped the nations of the modern world. □

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Superficial data

In today's increasingly automated world, one problem looms ever larger. How do you prove to a machine that you have a right to do something? It may be paying for a service, drawing money from a cash dispenser, travelling on a bus or train, entering a workplace, logging on to a computer, making a telephone call or many other things. The current solution lumps us with masses of cards, tickets and so on, often backed up by memorized codes as well.

Daedalus has a new solution: a machine-readable tattoo. A simple barcode on the wrist, say, might identify the wearer, but could never be updated or expanded, and would be very easy to forge or copy. So Daedalus proposes a magnetic tattoo, an area of iron oxide implanted into the skin. Iron oxide can be made in various shades of yellow and brown, cosmetically quite acceptable to most races (whites will be hardest to colour-match). Once in place, the tattoo could be written to, or read from, by standard magnetic methods.

This elegant scheme could encode all sorts of permanent or temporary authorizations. Name, date of birth, social-security number and blood group would be quite permanent. Credit-card data, driving-licence details, workplace status and magnetic pass keys might change from time to time. In addition, the tattoo could be used for all sorts of temporary or rapidly changing data. Its credit balance would alter every time the wearer bought anything with his tattoo, or paid money into his account with it. He could use it as a bus or train or theatre ticket, on which authorization could be written at the pay desk, checked at any time by officials, and erased at the exit. The masses of clumsy cards and tickets which now encumber our wallets would be completely outmoded.

Security would improve, too. You can't lose or steal a tattoo, nor can you easily photograph or copy a magnetic pattern. Even so, crucial financial data might best be protected by an update code. The computer reading or altering this data should always write a new check code on the tattoo and keep a note of it. A forger cannot know the latest code, so his forgery will always be out of date. Accidental erasure will be largely prevented by the intervening barrier of skin; in fact tattoo-coding equipment will need a special high-field writing head.

As the magnetic tattoo gains social and legal status, it may acquire ceremonial value as well. Daedalus has visions of the marriage of the future, in which bride and groom have their marital status ceremonially updated; and the ritual humiliation of bankrupt businessmen or disgraced officials by public erasure of their credit limits or workplace-status numbers.

David Jones

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Dinosaur eggs from Romania

SIR—A recent rock fall at an outcrop of Late Cretaceous age (65 million years ago) in the northern part of the Hateg Basin of Transylvania, Romania (4.5 km east of the town of Vălioara) has revealed the first dinosaur eggs to be found in Romania. Fourteen eggs, distorted to varying degrees, were discovered in a 0.5-m-thick layer of red, silty, clay that has since been found to contain fine volcanic

tubercles mark the external ends of densely packed and wedge-shaped, prismatic crystalline units that do not interlock laterally. Under high magnification they can be seen to be made of fine laminae which lie parallel to the external surface of the shell. These structures correspond closely with those described as tubocanaliculate^{1,2}. In vertical section the internal surface of the shell is irregular, following the bases of the crystalline units.

The identity of the animals that laid these eggs, as well as eggs of similar age from southern France, is the subject of debate^{1,2}: the Hateg fauna includes ornithopod, ankylosaurian, theropod and sauropod dinosaurs, pterosaurs, crocodiles, turtles, birds and primitive mammals (multituberculates)^{3,4}. The large diameter of the eggs (150 mm) excludes as potential layers all except the larger known elements of the fauna: "*Titano-saurus*" (= *Magyarosaurus*) (a sauropod), *Telmatosaurus* (a hadrosaur), and a newly discovered large theropod (D.B.N., D.B.W. and D.G., manuscript in preparation).

It has been proposed^{1,2,5} that tubocana-

liculate-type dinosaur eggshells were laid by sauropods: on this basis we conclude that these eggs were probably laid by a sauropod dinosaur, and should be referred to provisionally as *Magyarosaurus* eggs.

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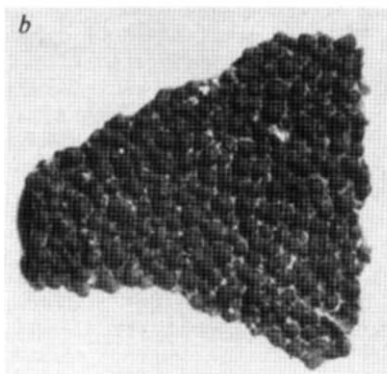
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a, A well-preserved egg attributed to "*Magyarosaurus*" collected from the locality near Vălioara (0.23 × natural size). b, External surface of the eggshell at low magnification to show the tuberculated surface (× 1.58).

ash and is sandwiched between iron-rich marls, with thin calcrete nodules and tubular burrows beneath. An erosional surface above the clay bed is topped by a thick bed of conglomerate of epi- and volcanoclastic elements in a tuffitic matrix.

The eggs are sub-spherical in shape and were found lying clustered in four linear rows, each comprising either two or four eggs. The distance between two clutches containing respectively two and four eggs was 0.5 m. Eight eggs are well preserved, with their lower surfaces almost complete, but the upper surfaces cracked and eroded (see figure). Shell fragments from the upper surface were found in the sediment surrounding the eggs. No skeletal remains were found.

The thickness of the eggshell varies between 2.3 and 2.4 mm. The external surface is irregularly tuberculated (b in the figure) and air canals between the tubercles are filled with calcite. The

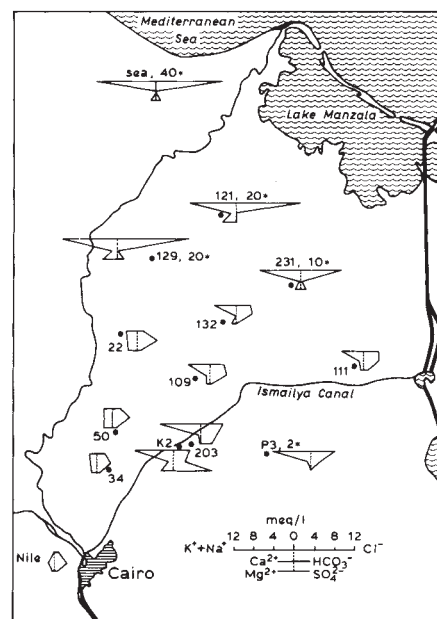
Ground water in the Nile delta

SIR—Burns *et al.*¹ suggest that increases relative to Nile water of the concentrations of sodium and potassium in ground water under Karnak (upper Egypt) may be caused by evaporation, and that the evaporation percentage is 89%, which they consider rather high. The calculation of evapotranspiration from concentration increases in ground water with respect to source water, is an old and simple technique^{2,3} that works well when the amounts of the ions are strictly conserved and their sources are known with confidence.

Anions such as chloride are often well conserved because, in many ground waters the only source of chloride is atmospheric deposition; chemical processes do not significantly affect concentration. But cations such as sodium and potassium are typically not conserved, because they are involved in biological cycles, are set free in weathering reactions of feldspars or participate in cation-exchange reactions. The large increase of sodium and potassium reported in ref. 1 could be caused by dissolution of Na-minerals from the soil in combination with cation-exchange.

Fresh ground water in the Nile delta originates almost exclusively from Nile water. Ground water along the Ismailia Canal, which carries water from Cairo to the eastern part of the delta, shows an increase of chloride-ion concentration with respect to Nile water undoubtedly caused by evapotranspiration. At the same time, the ratios of the main cations

change, as shown in the Stiff diagrams (see figure). The cations calcium and magnesium show a relative decrease, and sodium and potassium show a relative increase with respect to chloride. The decrease of calcium and concomitant increase of sodium could well be a result of dissolution of sodium minerals such as trona, and



Groundwater compositions in the Nile delta, Egypt, indicating saltwater upconing in boreholes 121 and 129, and Ca/Na-exchange near the Ismailia canal. Groundwater samples from the archives of the Groundwater Research Institute, Cairo.

cation-exchange in the sodium-containing loess continually blown in from the desert which settles in the wetter delta area. Such effects have been noted in the Negev desert⁴, and the coastal plain of Israel⁵.

Cation exchange is even more clearly apparent in salt water from boreholes 121 and 129, in which Ca is increased compared with sea water; this is typical of areas where overexploitation of fresh ground water has resulted in the upward movement of salt ground water. The cations from seawater (mainly sodium) then exchange with those from the freshwater sediment (mainly calcium) in a process that is a good indicator of salt water upconing even when detailed hydrological information is lacking⁶.

The message is clear: relative increases in groundwater concentrations can be used to calculate evapotranspiration, if the principles outlined by Eriksson are valid. This is difficult to demonstrate for cations such as sodium and potassium.

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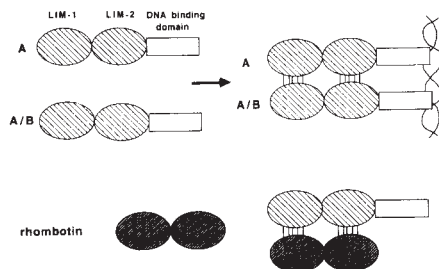
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LIM domains

SIR—In the previously reported protein sequence of the presumptive T-cell oncogene rhombotin^{1,2}, the presence of two tandemly repeated cysteine-rich regions (CRR) was noted, suggesting that these might correspond to metal-binding domains. We have now found that these CRR domains are equivalent to the recently proposed LIM domains in the nematode cell-lineage proteins lin-11 and mec-5 (ref. 3) and the vertebrate transcription factor Isl-1 (ref. 4). This similarity places the rhombotin protein in the family of proteins involved in transcription control. But although lin-11, mec-5 and Isl-1 share a putative DNA binding homeodomain as well as the LIM domains, there is no such DNA-binding domain in the rhombotin protein.

This difference in the putative protein structures, therefore, suggests that LIM domains facilitate, in this case via metal binding, protein-protein interactions in a manner analogous to the leucine zipper⁵ or the helix-loop-helix (HLH) motif⁶. Proteins carrying only protein oligomerization motifs could thus compete with transcription factors carrying compatible protein-interaction domains by docking with these proteins and preventing dimerization (see figure). The presence of proteins carrying only protein dimeriza-



Docking model for LIM domain interactions. The hypothetical protein A is capable of homodimer or heterodimer (or oligomer) formation with protein B, via metal linkage at the LIM domains, to form a complex specifically binding to DNA and involved in transcription. The formation of this transcription complex can be competed for by interactions at compatible LIM domains with LIM-only proteins. Activity of such complexes will depend on the balance of concentrations of the various involved proteins. Thus the combinatorial interaction of LIM-only and DNA-binding proteins represents a potential strategy to regulate gene expression.

tion motifs may not be without precedent: myoD carries an HLH domain and a basic region thought to be involved in DNA binding⁷, and Davis *et al.*⁷ cite unpublished evidence for the presence of a protein carrying only an HLH domain in the same cell lineage. Generalizing on earlier proposals⁸, interactions of this type could be one way to explain functional inactivation of genes by dominant negative mutations.

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Increased bloom

SIR—Algal blooms have recently received considerable attention, particularly when they cause mass mortality of marine organisms. A worldwide increase of algal blooms in coastal waters has been documented and is usually related to man-induced increase in nutrients. It is still open to question whether algal blooms in the North Sea have increased due to such a eutrophication. This debate has considerable political interest: are measures to decrease nutrient inputs to the North Sea necessary?

In the Southern Bight of the North Sea, the colonial flagellate *Phaeocystis* sp. is the most important bloom-forming alga. Fortunately, it is not toxic, but its intensive foam production during the wane of these blooms makes North Sea beaches unattractive during the tourist season¹.

Continuous plankton recorder (CPR) data² indicate that *Phaeocystis* around the British Isles decreased from 1948 to about

1970, since when it fluctuated at low levels to increase again slightly from 1980 onwards at the British side of the Southern Bight. For the Dutch side of the Bight the trend is similar, but since 1980 not enough CPR data have been available³.

This general decline and low levels of *Phaeocystis* after 1970 apparently contradict the almost continuous increase in duration of *Phaeocystis* blooms observed since 1974 at the Marsdiep station³ for northern Dutch coastal waters. Historical data on *Phaeocystis* in the Marsdiep⁴ indicate that *Phaeocystis* blooming periods lasted about 50 days in 1897 and 1899. The duration of these periods has increased two to threefold since then. These historical data and data for 1987–89 confirm our earlier conclusion of an increasing trend in duration of *Phaeocystis* blooms³.

These two conclusions do not contradict each other: different water masses were sampled in both studies. The towing depth of the CPR (10 metres) prevents any sampling in coastal waters, and the number of sampling routes in the North Sea has decreased. Waters near the Dutch coast have hardly been sampled by CPR, certainly not adequately in recent years.

Dutch coastal waters are eutrophicated by continental river water, particularly from the Rhine⁵. This nutrient-rich water remains near the coast. The change observed during the past few decades in nutrient composition of the coastal waters (increase in nitrogen and phosphorus, no such increase in silica) particularly favours the flagellate *Phaeocystis*^{3,5}. Whereas changes in plankton in the open North Sea largely reflect climatic changes², in Dutch coastal waters these climatic influences on phytoplankton seem to be overruled by eutrophication effects. Thus, *Phaeocystis* apparently behaves differently in Dutch coastal waters and in open North Sea water, in accordance with the respective elevated and virtually constant nutrient levels.

A decrease in nutrient loadings of the coastal waters is necessary to curb the ever-increasing algal blooms. Apart from nuisance due to foam accumulation on the beach, it is predictable that decomposition of the increased amounts of algal organic matter will lead to oxygen deficiencies, even in normally well-mixed Dutch coastal waters like the Wadden Sea.

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Water balance in the Black Sea

SIR—Murray *et al.*¹ suggest that the combined pycnocline/chemocline in the Black Sea has risen unexpectedly and at an alarming rate. Their argument rests on the comparison of the RV *Atlantis II* hydrographic station 1445 of March 1969, to the RV *Knorr* June 1988 station BS3-2, about 30 nm away in the western Black Sea basin. The two records showed a 0.1‰ increase in salinity between a depth of 50 and 100 m and a rise of the chemocline by 30 m. Murray *et al.* suggest that the water balance of the Black Sea has been upset by a decrease in discharge of Soviet rivers. To compensate, the input of Mediterranean water through the Bosphorus should have been higher. Indeed, a rough calculation of the water and salt balance based on this comparison does not exclude such an interpretation.

This interpretation, however, may be countered by field observations. For examples, stations Atlantis 1432 (23 March 1969) and 1463 (14 April 1969) were just a few miles apart but showed within three weeks a rise in the salinity profile of up to 80 m between 20 and 220 m depth, that is, a much larger rise than the one reported by Murray *et al.* (a in the figure). Second, our salinity measurements during the RV *Piri Reis* Black Sea cruises of 1984 show a rise of the pycnocline of 15 to 20 m between April and October (b). Third, during leg 1 of the *Knorr* Black Sea cruise (16 April–9 May 1988)², many conductivity–temperature–depth (CTD) stations were occupied (using the same Seabird CTD as Murray *et al.* and an independent instrument) throughout the entire southern Black Sea.

Nine of these stations can be compared with nearby former Atlantis stations. Seven of them show a higher interface in 1988 than in 1969, but two stations from the eastern Black Sea show a much deeper interface (c). Thus the interface seems to be intensively warped, possibly more pronounced in April 1988 than in March–April 1969. A net long-term rise cannot,

however, be ascertained, at least not without mapping the interface throughout the entire basin simultaneously. Finally, we also occupied stations near the coast twice within a few hours and observed that the pycnocline moved up and down by several tens of metres between casts. These movements warp the chemocline along the coast and are associated with features of the coastal currents or with internal waves travelling along the pycnocline.

Variations in chemo- and pycnocline depths are strongly coupled. Our measurements of dissolved phosphate in May 1988 show upward movements similar to those observed by Murray *et al.* in the western Black Sea. In the eastern basin a depression of about 30 m was found relative to that at Atlantis station 1463. The structure of the manganese particle layer, which is found at the +100 mV level in the redoxcline (and associated with a density of 16.1–16.3σθ) may be indicative of the prevalent transport processes. The layer is deeper, more intense and thicker near the coast. It is often split into several sheets, each measuring a few metres in thickness only.

These sheets may represent layers of the water advecting laterally with different velocities. They could transport cold intermediate water towards the centre of the gyres causing a rise of the pycnocline offshore while at the same time warm and low-salinity surface water is displaced from the centre towards the margins of the Black Sea. Such a change could come about by more intensive coastal surface currents triggered by winter storms. This model would also fit the observations, suggesting lateral transport of resuspended shelf sediments within the upper 200 m of the water column and indicating radical changes in the annual vertical particle flux in the upper part of the water column^{3,4,5}.

We therefore believe that the observed differences between 1969 and 1988 are associated with interannual changes in the intensity of Black Sea surface currents

and with internal waves rather than with anthropogenic changes of the Black Sea water balance.

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Random thoughts

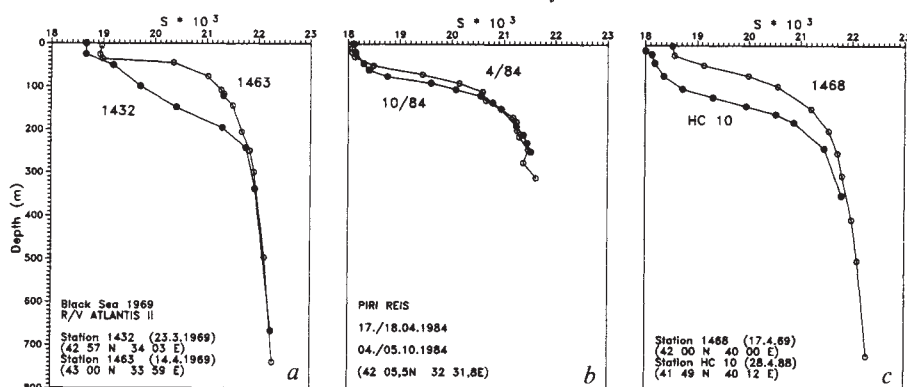
SIR—A minor slip in Hubert P. Yockey's most informative letter "When is random random?" (*Nature* **344**, 823; 1990) requires clarification. He writes: "Thus it is clear that pi and all other transcendental numbers have a finite amount of information, namely, that in the algorithm by which they are calculated." From this statement one would infer that every transcendental number may be calculated by some algorithm. But there are only countably many algorithms and uncountably many transcendental numbers. Hence one cannot conclude from Yockey's argument that each transcendental number contains only a finite amount of information. This issue is, of course, only incidental to the point of his letter.

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SIR—The discussion about the meaning of 'random' needs to take into account two separate uses of the word. Information-theoretic or algorithmic approaches to measuring the randomness in a sequence of digits may be appropriate after the sequence has been generated or defined, but there is also a sense in which a process may be agreed to be random before it has been used to generate any sequences whatever. A sequence produced by a random process is not necessarily random.

Yockey (*Nature* **344**, 823; 1990) blurs the distinction by using the name 'Bernoulli chains or strings' for the actual sequences of heads and tails (say) generated by what probabilists have long called 'Bernoulli trials'. It is not necessarily true that '[i]n the case of a sequence of heads and tails generated by



a, Comparison of salinity between two RV *Atlantis II* stations showing the rise of the pycnocline by up to 80 m within about 3 weeks. b, Comparison of salinity profiles of RV *Piri Reis* cruises in April 1984 and October 1984. c, Comparison of salinity profiles (Atlantis 1468 with Knorr BS1 HC 10).

the tossing of a fair coin, there is no algorithm which can shorten the sequence' because the sequence might be all heads, for example. Bernoulli's *Ars conjectandi* contained no discussion of actual sequences, and his name should surely be reserved for the generating process.

But, as Yockey goes on to say, "It is impossible to prove that any given sequence was generated by a random process"; in other words, it is impossible to prove that a process is random by examining its output sequence. Rather, a process is called random if its structure ensures that its output is unpredictable.

Statisticians are familiar with the separate uses. A good experimental design will need to be based on the first kind of randomness lest it display systematic bias, but a randomized trial will need the second kind so that the participants cannot guess what is coming next.

Whether randomness can be measured is a difficult problem (*Nature* **344**, 705; 1990). One cannot judge the absence of pattern without specifying which pattern, and what is a pattern to you may not be a pattern to me.

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Superconductor defect structure

SIR—One of the main obstacles to the practical use of high-temperature superconductors is that the amounts of current that they can carry and still remain superconducting (that is, their critical current densities) are relatively low. A processing method that has significantly increased the intragranular critical current density involves the solid-state phase transformation of single-phase $\text{Y}_2\text{Ba}_2\text{Cu}_3\text{O}_{10}$ (commonly referred to as '124' or '248') into $\text{YBa}_2\text{Cu}_3\text{O}_7$ ('123') with the concomitant formation of a heavily defective microstructure. Here we report a defect structure of apparent composition $\text{YBa}_2\text{Cu}_5\text{O}_9$ ('125') in solid-state-transformed sintered bulk samples of 124.

The decomposition of 124 precursor was carried out by heat treating it at 930 °C for 1.5 min followed by a low temperature oxygenation process of rapidly cooling (10 s) to about 750 °C with further cooling to 380 °C at 20 °C h⁻¹ in flowing oxygen. Further details of sample preparation are given elsewhere¹. The defect structure was examined by high-resolution transmission electron microscopy (HREM) using the Berkeley atomic-resolution microscope. Thin foils were prepared by mechanically grinding a slice of the superconductor to about 50–80 µm, followed by argon-ion milling

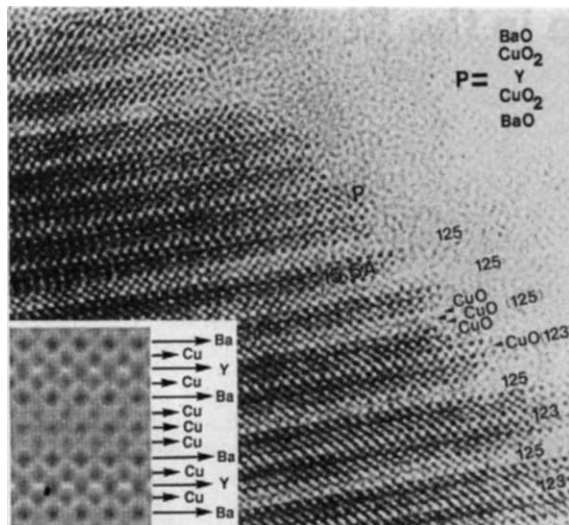


FIG. 1 Atomic-resolution image of the phase-decomposed 124 sample illustrating the 123 structure alternating with the new 125 defect structure. Layers of 125 two or three unit cells thick are seen in the upper and middle part of the micrograph. The simulated intensity distribution for the cation positions (inset) of 125 for a foil thickness of 12 Å and an objective-lens defocus of -500 Å, confirms the interpretation of the contrast in the micrograph.

to electron transparency at 6 kV and 77 K. Images were obtained under conditions close to Scherzer defocus and the projections of atomic columns therefore appear as dark dots on a white background. To avoid electron-beam-induced damage, images were typically recorded within 2 min of exposure to the beam.

A micrograph of the defect structure (Fig. 1) shows the decomposition of 124 into 123 and 125. The structures of the three related phases are illustrated in Fig. 2 (a–c). Analysis of the image contrast using detailed image simulations indicates that the basic perovskite building blocks (marked P in Fig. 1) in the 125 structure are similar to those in the 123 structure, except that the 125 structure has three CuO chain layers whereas 123 has one. The 124 structure has two CuO chain layers with an $a/2[100]$ glide plane between

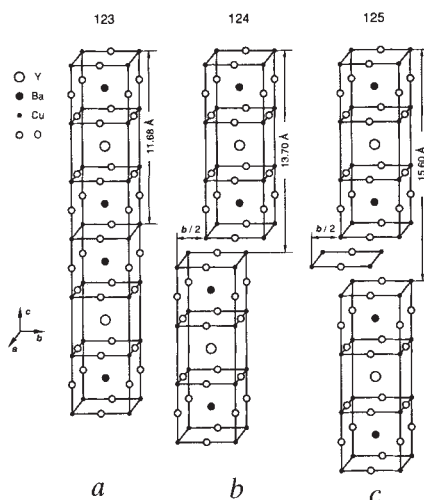


FIG. 2 Schematic illustrations of the structure 123, 124 and 125.

them^{2,3}. In the proposed 125 structure (most likely $\text{YBa}_2\text{Cu}_5\text{O}_9$) the three CuO chains are mutually related by a $1/2[100]$ glide operation and hence, unlike 124, there is no doubling of the unit-cell dimension along the c direction. The c -axis lattice parameter is expected to be 15.6 Å, based on the addition of one more CuO chain layer to the 124 structure (the Cu–O distance along the c axis is about 1.9 Å, as established by neutron diffraction and HREM analysis³). In general, single layers of 125 alternate with single layers of 123 but occasionally layers of 125 two to three unit cells thick occur.

The fact that the 123 and 125 layers are formed as alternating layers with very rapid kinetics suggests that the early stage of the

decomposition of 124 may occur by a spinodal (spontaneous decomposition without a nucleation barrier) process. Such a decomposition to 123 requires only short-range diffusion along the c direction (jumps over atomic-layer distances), as can be seen from Figs 1 and 2. During the later stage of decomposition, however, nucleation and growth reactions seem to take over, involving the removal of copper and oxygen by long-range planar diffusion to form equilibrium, twinned 123 and spherical CuO inclusions.

So far, our attempts to synthesize the 125 phase as a single phase in bulk form by conventional ceramic processing have failed. Perhaps the formation of the 125 phase is easier when the 124 phase spontaneously decomposes into the 123 structure, forcing the formation of the 125 structure. We note that recent three-dimensional Monte Carlo simulations using experimental thermodynamic data predict that a bulk 125 containing material can be synthesized at a very high oxygen pressure in a copper-rich environment (M. Fendorf, personal communication).

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Diversity and differences

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Transmembrane Signalling. Intracellular Messengers and Implications for Drug Development. Edited by Stefan R. Nahorski. Wiley: 1990. Pp. 248. £39, \$76.

G-Proteins as Mediators of Cellular Signalling Processes. Edited by M. D. Houslay and G. Milligan. Wiley: 1990. Pp. 232. £35, \$72.45.

TRANSMEMBRANE Signalling presents papers from a meeting held in the spring of 1989. As a symposium volume, it displays all the distinguishing marks of the genre. It is out of date. It promises more than it delivers. It neglects fundamental questions in favour of reciting facts collected in individual laboratories. More specifically, of fourteen chapters, nine are devoted to a single mode of transmembrane signalling, via G proteins and their confederate receptors and effectors; the remaining five focus on a single network of intracellular messengers, the Ca^{2+} /phosphoinositide cascade. The book's title suggests that it might have accorded to receptor tyrosine kinases more than the offhand comment in chapter 8, and to cyclic nucleotides more than the four pages on synthetic cyclic AMP analogues in chapter 14. Moreover, its pages provide not a single example of the "implications for drug development" advertised in the subtitle.

False advertising might be forgiven if the product were more timely or more interesting. Most chapters, however, contain little more than accounts of the research of one laboratory, about two years ago. Of the book's first 234 references (after which I stopped counting), only five cite papers published later than 1988. Sadly, detailed findings of a single laboratory — even a good one — do not become more fascinating as the years pass. In mid-1990, only a few chapters — including those by Birnbaumer *et al.* (on G proteins and ion channels), Irvine (inositol polyphosphates) and Pouyssegur *et al.* (G proteins and cell proliferation) — are sufficiently thoughtful, provocative or comprehensive to merit attention. Note, however, that more recent reviews of the same subjects, in some cases by the same authors, are available at much lower cost, by taking the time to peruse a few current journals on library shelves.

Rather than embalming a venerable symposium, the editors of *G-Proteins as Mediators of Cellular Signalling Processes* have put together the first in a projected series of scholarly multi-author books on the "molecular pharmacology of cell regulation". In this case the book's title correctly describes its contents, which ought to be relevant to many biologists in an era when one can hardly open a journal without meeting GTP. Several chapters provide exactly what one seeks in a short monograph. Hall comprehensively surveys

recent literature on Ras proteins and their relatives, making more than the usual amount of sense out of a plethora of diverse and sometimes contradictory experimental observations in many laboratories. Hingorani and Ho do the same for the well-studied G protein of retinal photoreceptors, transducin; their bold representations of relations between transducin structure and function will provoke thought and experiment, even if some predictions turn out to be wrong.

In general, however, the book is not appreciably more up to date or comprehensive than the symposium volume. Again, references after 1988 are rare, and too many chapters concentrate on recounting experimental findings in their authors' own laboratories. Self-citations account for more than a quarter of the references in both books. The narrow focus characteristic of symposium contributions is especially inappropriate in scholarly summaries of a complex field.

G-Proteins would have profited from a more assertive editorial hand. The editors should have corrected obviously erroneous statements (for example, the attribution, on page 1, of a single transmem-

brane region to catalytic adenylyl cyclase), as well as the typographical errors found on almost every page (such as the "autonomic" functions of transducin, on page 101). They should have deconvoluted pompous, obscure prose in several chapters (see page 86 for a rich assortment of abominable sentences). They should have blue-pencilled the boring, repetitious accounts of G proteins that introduce every chapter. Finally, far too many chapters portray a motley army of lonely facts — gel filtration profiles, immunoblots, amino-acid sequence motifs and graphs of something versus something else — wandering mindlessly across the page in search of an idea. The editors could have produced a much more interesting and readable book.

Every scientist can name at least one collection of scholarly reviews (even, perhaps, a symposium volume) that challenged active investigators, recruited new ones and enriched a field. But in many fast-moving research areas, the quality of most such tomes is negligible and likely to remain so. The root of the problem is not greedy publishers but scientists themselves. Some of us covet cheap air travel, paid for with a manuscript, while others just yearn to see our names in print. Who among us has not scribbled a rehash of past exploits without bothering about whether anyone will (or should) read it? We have met the enemy, and he is us. □

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Stenopus hispidus, the banded coral shrimp, is the most distinctive of the shrimps common to the reefs of Hawaii. Taken from *Hawaiian Reef Animals* by E. Hobson and E.H. Clave, first published in 1972 and now reissued in a revised edition. The volume describes the vertebrate and invertebrate communities of the Hawaiian reef, and contains 85 colour plates of the variously decorated animals of the reef. Published by University of Hawaii Press, pbk \$19.95. □

New in paperback

■ A new volume in the "Practical Approach" series published by IRL Press at Oxford University Press is *Ribosomes and Protein Synthesis*, edited by G. Spedding. Price is £22.50 (also available as spiral bound hardback at £32.50).

■ *Phonological Skills and Learning to Read* is by Usha Goswami and Peter Bryant. The authors discuss recent research on reading strategies adopted by children and develop a theory of phonological awareness as a precedent of reading. Publisher is Erlbaum, price is £9.95 (hbk £19.95).

■ Urwin Hyman has recently published *Marine Geochemistry* by Roy Chester. The book offers a fully comprehensive summary of the chemistry of the oceans, their sediments and biota. Written as a textbook for advanced students, price is £34.95 (hbk £90).

■ New in paperback is *The Cambridge Illustrated Dictionary of Natural History* by R. J. Lincoln and G. A. Boxshall. Published by Cambridge University Press in hardback in 1987, the book contains 10,000 entries and more than 700 illustrations. Price is £9.95, \$14.95.

■ *Stars and Galaxies. Citizens of the Universe* edited by Donald E. Osterbrock is a recent addition to the series "Readings from Scientific American". The collection presents research on galaxies, luminous stars and supernovae. Publisher is Freeman, price is £10.95.

■ *The Path of No Resistance* is subtitled *The Story of the Revolution in Superconductivity*. Published by Simon and Schuster as a Touchstone paperback, the author is Bruce Schechter, price is \$8.95. □

Seeds of the past

Benno Müller-Hill

The Wellborn Science. Eugenics in Germany, France, Brazil and Russia. Edited by Mark B. Adams. Oxford University Press: 1990. Pp.242. £35, \$45.

For a long time, eugenics has been forgotten by historians of science. Now, when almost all the participants are dead — and just before the Human Genome Project becomes a reality — their interest in the subject is re-emerging. So far, all the books on the subject have concentrated on eugenics in just one country. This one, edited by Mark Adams, differs in that it is the first which attempts an international approach. Four authors deal with four countries: Germany, France, Brazil and the Soviet Union. Four authors are necessary, according to Adams, because scholars in the United States know at best one language in addition to their own. So the approach is necessarily fragmented. Nevertheless, only one-tenth of the countries in which eugenicists were active are dealt with here.

The difference between what has been called eugenics in each of these four countries is tremendous. On one end of the scale is Brazil, where not a single researcher ever actually worked on genetics until 1940, the year the study ends. Eugenics in Brazil was a mixture of sport and hygiene. Nobody there ever thought seriously about sterilization, and nobody was upset by race mixing — everybody optimistically expected the "whitening" of the inhabitants. The article by Nancy Leys Stepan confirms what one may have guessed: eugenics in the absence of geneticists cannot have a strong impact on society.

Strangely enough, according to William Schneider, France was not so very different from Brazil. Before World War I eugenicists in France were inspired more by Lamarck and by Zola's novels than by Mendel. According to Schneider only one geneticist, Cuenot, was active in France before 1930. But the ultimately hopeful world of Zola collapsed with World War I, and a new generation of eugenicists appeared in the 1930s. Curiously, Schneider does not mention the French Nobel prizewinner Alexis Carrel, who proposed the "ideal solution" of "little economic gas chambers" for all those with bad genes in his book *L'homme cet Inconnu* (Plon, 1935). Schneider's account stops at the moment where it would have become the most interesting with the collaboration between the French and the Nazi authorities in 1940. So the Swiss anthropologist Montadon is men-

tioned as "never subscribing to the possibility, let alone the advantage of achieving racial purity". Montadon certainly changed his opinion in his tract "What is a Jew?", which was published during the German occupation and has just been reprinted in France.

Adams paints a fascinating picture of the development of eugenics in the Soviet Union, where the subject was strongly associated with some outstanding mendelian geneticists. Kol'tsov, Filipchenko and Serebrowsky understood and practised genetics. Kol'tsov, who wrote in 1922 "Eugenics is the religion of the future and it awaits its prophets", was an optimist who could not have imagined somebody like Hitler. To propose mass sterilization was out of the question in this devastated country, so Serebrowsky, and later H. J. Muller, proposed sperm donation. But with the rise of Stalin, genetics and eugenics disappeared with the geneticists.

The other country considered here in which geneticists were strong and eugenics was flourishing, Germany, is dealt with by Sheila Weiss. Her article has to compete with Weindling's voluminous book *Health, Race and German Politics 1870-1945* (Cambridge University Press; for review see *Nature* 344, 502; 1990) and does so very well. Germany was the only country where eugenic measures were applied on a vast scale by the government. Weiss concentrates on the different aspects of the various breeds of eugenics. The details of the Nazi variety as propagated by various state agencies are somewhat neglected. But she shows convincingly how just one brand of eugenics was selected by the Nazis and brought into action.

At the end of the book the reader is left with some nagging questions: why was eugenics so different in different countries? Why were eugenicists successful only in Germany? Could some other form of the practice become successful in the future? The reader is reminded by Adams in the concluding chapter that the book deals after all with only four of about forty countries in which eugenicists have flourished. And if the past is so complex, what about the future? I think this volume should be bought and read not only by historians of science but by all those interested in and involved in the Human Genome Project. To know what was thought and what happened in the twentieth century may be helpful for those who will be active in the twenty-first. □

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■ In his last book review for *Nature* (336, 721; 1988), Professor Müller-Hill reviewed *The Bison* by Danil Granin, a novel about Nikolai Timofeyev-Resovsky, the scientist who defied Stalin. Just translated into English by A. W. Bouis, the book is now published in the United States by Doubleday, price \$24.95. □

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Business plan

Bob Johnstone

Computers Inc. Japan's Challenge to IBM. By Marie Anchordoguy. *Harvard University Press*: 1990. Pp. 273. £19.95, \$25.

INDUSTRIAL policy can work. That is the inescapable conclusion of this important new study on the rise of the Japanese computer industry. Classical economic theory — as preached by zealots of the Bush administration — states that governmental attempts to 'pick the winners' inevitably distort the market, ending up by doing more harm than good. Yet, as the Japanese have demonstrated, and as this book documents, an industry can indeed be nurtured and brought to international competitiveness. What it takes is a consistent, long-term strategy and close cooperation between public and private sectors. Proof, if proof is required, of Japan's success in the computer industry is evident in the admission by Ed Lucente, president of IBM's Asia-Pacific group, that IBM views Japanese computer companies as "our most serious competitors".

In 1960, IBM obtained permission from Japan's Ministry of International Trade and Industry (MITI) for permission to manufacture in that country. Back then, only brave souls would have predicted that, within two decades, Japanese domestic manufacturers would catch up with and — at least in some respects — overtake the giant US firm.

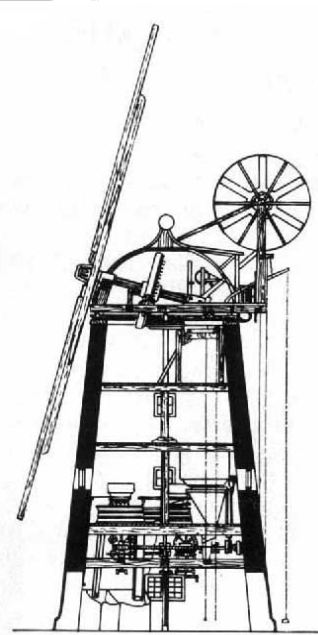
But that is exactly what happened. In 1980, Fujitsu — Japan's leading computer manufacturer — became the first company in the world to top IBM in computer sales. Although IBM remains the dominant force in the worldwide computer industry, with an estimated 70 to 80 per cent share of the \$40,000 million mainframe market, the Japanese are eroding the US company's market and technological leadership (see last week's News section, page 307). In June this year, for example, Hitachi, Japan's other maker of IBM-type mainframe computers, announced that it would shortly begin sales of machines considerably faster than IBM's current top of the range models. Less than a month later, Hitachi's rival NEC produced a mainframe that it claims is even faster than Hitachi's.

The Japanese are adept at using the threat of foreign dominance — real or imagined — to motivate themselves to redouble their efforts in any given endeavour. Of all the bogeymen they use, IBM has traditionally been the most frightening.

IBM's request for permission to produce locally seems to have reminded MITI bureaucrats of the arrival in Tokyo Bay,



Early windmill design, from *The Traditional Buildings of England* by Anthony Quiney. Published by Thames and Hudson, price £14.95.



about 100 years earlier, of Commodore Perry with his fleet of black ships, demanding that Japan open up to foreign commerce. MITI officials determined that the giant US firm would not be allowed to dominate the Japanese market as it had — in their estimation — in Europe. To counter IBM, says Anchordoguy, "Japan would turn to its full repertoire of industrial policy tools". These included protectionism, appeals to patriotism and outright coercion. Fiscal tools included subsidies, tax-benefits, low-interest loans and loan guarantees.

It is often thought that the Japanese government merely sprinkles financial aid on infant industries, leaving companies to foot most of the bill. In the case of the computer industry, however, as Anchordoguy is at pains to point out, this was certainly not true. "A conservative estimate shows", she writes, "that some US \$542 million in subsidies, tax benefits, and loans were granted to the industry from 1961 to 1969." This figure does not include "the huge benefits of procurement from NTT" — Japan's public telecommunications monopoly, which played a very important complementary role in the development of the Japanese computer industry — "and other government institutions."

Perhaps the most significant, and certainly the most innovative, support mechanism that MITI came up with to boost sales of computers was the Japan Electronic Computer Company. This was a government-funded company which between 1961 and 1981 purchased computers worth \$2,000 million from the makers, then rented them to users. The company had the dual purpose of providing companies with cash that they could quickly plough

back into development, while at the same time stimulating demand by making computers relatively easy for users to afford.

But MITI also made some false moves. Most notable among them was the Japan Software Company, a joint venture it created to provide programmes for computers developed under a ministry project. Isolated from the market, the firm failed to produce software that anybody wanted, and was dissolved in 1972.

For the most part, though, things went according to the ministry's master plan. One important factor that helps to account for the success of MITI's strategy was that it managed to achieve a workable blend of cooperation and competition between firms. Japan was thus able to avoid the pitfalls of the single-company 'national champion' approach of Britain and France.

Companies were happy to make the best use of scarce resources by collaborating on upstream development efforts. Nor did they object too strenuously to being paired off (Fujitsu with Hitachi, NEC with Toshiba and Mitsubishi with Oki) to attack different market segments, thus avoiding excessive competition. In their individual segments, however, companies competed fiercely against one another. And, despite generous government subsidies, all were prepared to commit significant investments of their own.

Others have told the story of the development of the Japanese computer industry before. But no-one has told it so well, or in so much detail, as Anchordoguy in this excellent book.

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View from the shore

Simon Conway Morris

Terrestrial Invasion. An Ecophysiological Approach to the Origins of Land Animals.

By Colin Little. Cambridge University Press: 1990. Pp. 304. Hbk £40, \$69.50; pbk £13.95, \$29.95.

ONE of James Lovelock's most compelling images is that of the early Earth, not as an azure jewel hanging in the blackness of space, but a dirty red smog-bound ball. Such invocations haunt us because they provide the mainspring to catapult our imaginations into the distant geological past. How, then, would a time-traveller see our planet a mere 450 million years ago, towards the end of the Ordovician? An atmosphere now cleansed by the purifying action of oxygen, deep blue oceans and polar ice-caps, but the continents themselves dun and barren, devoid of plants — except, perhaps, along the fringes of the sea, where a green tideline heralded a biological revolution. The invasion of land was on, and here is a scholarly book to guide us as to how this might have been achieved.

It is a story that demands grand themes, but the reader will have to work pretty hard to find them among all the minutiae. Most striking, yet most neglected, is just how dynamic terrestrial invasion has been. For too long it has been construed as a largely Palaeozoic affair. The evidence is, however, that some animals were creeping onto land much later, in the Cretaceous or even in the Tertiary. These animals might have included the decapods and littorinacean snails. Indeed, the evolution of entire shoreline communities, especially the mangrove swamps and saltmarshes in the Cretaceous, may have done much to facilitate successful terrestrial transfer as well as those making the return journey to the sea. Ecological analogues to the mangrove swamps earlier in geological history are decidedly tenuous, so here is a timely warning about the inapplicability of uniformitarian ecology.

Meanwhile, the invasion of land probably continues. Little's suggestion that diversity of a group could be linked to time of invasion might mean that the taxonomically depauperate terrestrial amphipods may have come ashore only in the past few million years. Moreover, many of the stages of terrestrial invasion are still evident from the varying abilities of shore-dwellers to cope with the rigours of the

subaerial habitat. In some, relative success is marked by physiology, but simple rules of thumb are elusive and factors such as behaviour are often significant.

Another theme is to identify the main routes of invasion. Great stress is laid on physiological evidence. For example, terrestrial groups with either osmotic tolerance or high osmotic pressure are taken to indicate a direct marine origin. There are some alarming exceptions. In teleost fish, blood composition indicates a freshwater phase, although why marine representatives still carry this apparent signature is far from clear. The book is peppered with such examples of wayward ecology and physiology where no satisfac-

group after group grappled with the tremendous changes that assault the terrestrial invader. These are most striking in terms of respiration, take for example the remarkable 'lung' of the crab *Ocypode*, and sensory perception as emphasis shifted from chemical to visual cues. But what is urgently needed is a phylogenetic perspective to this welter of ecological and physiological data. The fossil record may be appalling (although, curiously, Little does not mention the amazing finds such as those from the Devonian deposits at Gilboa), but detailed phylogenetic testing is still feasible.

So here is an excellent compilation which, if somewhat naive in its geological

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Historical view of animals' attempts on the land.

tory explanation exists, and although Little is properly cautious there is a sense of the *ad hoc* about some proposals. Take also the potentially fascinating cases of failures in the invasion of land. For no obvious reason the gecarcinid crabs failed to penetrate inland, and despite their adroit subaerial excursions, the mudskippers apparently have not given rise to terrestrial lineages.

Little has much of value to say on the environments of invasion. He argues that beaches were a plausible route, with the severity of the transitional zone acting as a testing ground for would-be terrestrial aspirants. In the case of the vertebrates he casts further doubt on the time-honoured notion of proto-amphibians lurching from one drying pool to another, and suggests our terrestrial origins lay in stagnant freshwater swamps where oxygen crises imposed the need to switch to air-breathing.

This book is a rich lode of information, and it will be a shame if evolutionary biologists overlook some of its treasures. In particular, it represents a marvellous quarry for students of convergence as

sections, is unrivalled in its breadth and gathering of examples. It represents serious scholarship, and will be a source to all who seek to understand how it is that the land teems with life. But the main themes in terrestrial invasion, those that must inspire the next generation, still await their biological Boswell. □

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Palaeontological paperbacks

■ Recently published by Wiley is *Sleuthing Fossils. The Art of Investigating Past Life* by Alan M. Cvanara. The book contains fossil identifications and describes how to collect, identify and interpret fossils. Price is \$12.95.

■ *The Life of a Fossil Hunter* by Charles H. Sternberg was first published in 1909. Now reissued by Indiana University Press, Sternberg recounts vivid tales of the dangers of collecting dinosaur fossils in the wild west. Price is \$12.95 (hbk \$29.95).

■ New from Unwin Hyman is *Trace Fossils. Biology and Taphonomy*, part of the series Special Topics in Palaeontology, by Richard Bromley. Price is £17.95. □

Adhesion receptors of the immune system

Timothy A. Springer

The adhesive interactions of cells with other cells and with the extracellular matrix are crucial to all developmental processes, but have a central role in the functions of the immune system throughout life. Three families of cell-surface molecules regulate the migration of lymphocytes and the interactions of activated cells during immune responses.

THE organization of animal cells in differentiated organs and tissues has long been postulated to depend on cell-surface interactions both with molecules on the surface of other cells and with the extracellular matrix^{1,2}. Localization of cells can thus in principle be driven by the interplay between interactions with cell-surface and matrix molecules that regulate adherence, and chemoattractant gradients that direct cell migration. These principles operate throughout the biology of multicellular organisms^{2,3}, and the cells of the immune system in particular depend upon regulated interactions with other cells to activate and direct the response to infection. The distinctive antigen-specific interactions studied by immunologists, and the cell and tissue-selective adhesive interactions studied by cell biologists, have however traditionally found little common ground. This has changed recently as studies of the interactions of T lymphocytes with antigen-bearing cells have yielded not only the antigen-specific receptors, but also a harvest of cell adhesion molecules. Homologous families of adhesion receptors reveal relationships between molecules in the immune system and many other tissues, and participate in the control not only of cell-cell interactions but also in the regulation of cell migration.

The mechanisms for regulating adhesion, and their functional importance, are particularly richly illustrated by the adhesion receptors of the immune system. To patrol the body effectively for infectious organisms, the cells of the immune system must both circulate as nonadherent cells in the blood and lymph and migrate as adherent cells through tissues; in the presence of a foreign antigen, they must be able to congregate in lymphoid organs, cross endothelial and basement membrane barriers to aggregate at sites of infection, and adhere to cells bearing foreign antigen⁴. Rapid transition between adherent and nonadherent states is of key importance to the dual functions of immune surveillance and responsiveness. In this review, I shall explain what is known of the adhesive interactions that take place when lymphocytes have been activated by foreign antigen and that direct their localization and migration, before describing receptors that determine lymphocyte homing to different lymphoid organs and neutrophil localization in inflammation. Three families of adhesion receptors mediate these interactions (Figs 1–3): the immunoglobulin superfamily, which includes the antigen-specific receptors of T and B lymphocytes; the integrin family, which is important in dynamic regulation of adhesion and migration; and the selectins, which are prominent in lymphocyte and neutrophil interaction with vascular endothelium. Many adhesive molecules play a part in more than one of these functions, and some of them are exploited as receptors by viruses.

Antigen-specific recognition by T lymphocytes

Most of our knowledge about the molecules regulating lymphocyte adhesion has come from the study of T lymphocytes, whose functions in immunity depend on close contact with other cells. The T-cell receptor (TCR; Fig. 1) recognizes antigen as a peptide fragment bound to cell-surface molecules encoded by the major histocompatibility complex (MHC; Fig. 1). T lymphocytes are activated by specific binding of their receptors to peptide-MHC complexes on the surface of antigen-presenting

cells such as macrophages. This stimulates proliferation and differentiation of two types of functional T cells: helper cells, which promote the proliferation and maturation of antibody-producing B lymphocytes; and killer cells, which lyse cells infected with intracellular parasites such as viruses. Helper T cells recognize MHC-antigen complexes on the surface of the B cells whose proliferation they induce; killer T cells recognize complexes of viral peptides with MHC on the surface of the infected cells they kill.

The MHC molecules that present peptide antigens for recognition by TCRs fall into two structurally distinct classes (Fig. 1). MHC class I molecules bind to peptides derived from endogenously synthesized molecules such as viral proteins in infected cells and are primarily recognized by killer cells (Fig. 4a). MHC class II molecules bind to peptides derived from endocytosed antigen, and are recognized primarily by helper T lymphocytes (Fig. 4b).

All of the molecules now known to participate in the recognition by T cells of their targets were originally identified by monoclonal antibodies against cell-surface molecules of T lymphocytes. Two of the molecules so identified, CD8 and CD4, have since been shown to act as co-receptors respectively for class I and class II MHC molecules^{5,6}. The T-cell receptor and the co-receptors diffuse independently in the plane of the T-cell membrane until they are brought together by co-recognition of the same peptide-MHC molecule complex (Fig. 4a, b). At physiological densities on T lymphocytes and in the absence of antigen, CD4 and CD8 mediate little or no adhesion to MHC^{7,8} although overexpression in transfected fibroblasts has demonstrated binding by CD4 to class II and CD8 to class I^{9,10}. The main physiological importance of these molecules is in signalling: when their contribution is blocked by antibodies, T cells require 100-fold higher concentrations of antigen to induce responsiveness^{5,11–14}.

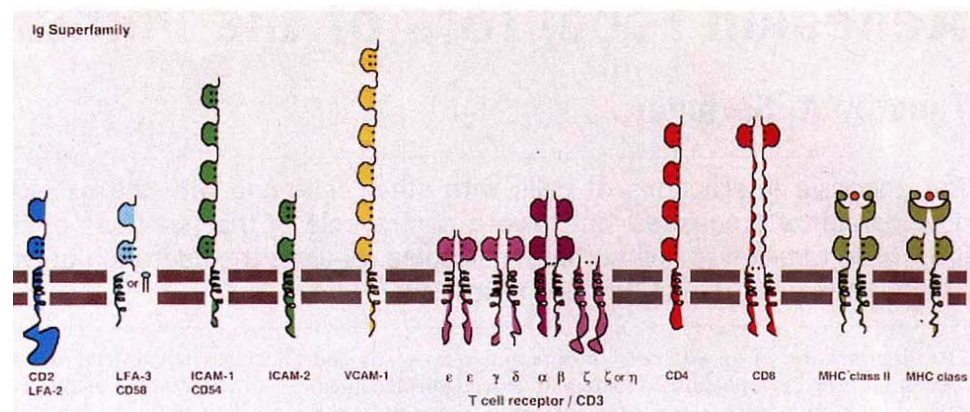
Synergistic signalling by the association of the TCR with its co-receptor has been directly demonstrated for CD8 (ref. 15), which has been shown to bind to the membrane-proximal domain of the MHC class I molecule that is not polymorphic^{15,16}, whereas the TCR binds to the polymorphic membrane-distal domains¹⁷. The evidence on CD4 is less direct; but when CD4⁺ helper cells form conjugates with antigen-presenting B cells, both the TCR and CD4 redistribute to the site of adhesion¹⁴, and antibodies that induce the association of CD4 with the TCR also activate T cells^{11,14}. The signalling function of CD4 and CD8 may depend on the association of their cytoplasmic segments with a lymphocyte-specific tyrosine kinase encoded by *lck* (ref. 18).

CD4 and CD8 may play a critical part in the differentiation of T-cell precursors in the thymus. Early in thymic ontogeny, immature T cells express both CD4 and CD8; coassociation of one or the other of these molecules with the T-cell receptor may signal to the thymocyte whether its TCR recognizes class I or class II MHC, and thereby regulate subsequent differentiation into CD8⁺ cytotoxic or CD4⁺ helper T-lymphocyte subsets^{11,12}.

Activation-dependent adhesion mechanisms

The definition of other cell-surface molecules essential for the

FIG. 1 Immunoglobulin superfamily adhesion receptors. Members of the superfamily share the immunoglobulin domain, composed of 90–100 amino acids arranged in a sandwich of two sheets of anti-parallel β -strands, which is usually stabilized by a disulphide bond at its centre^{136,137}. The immunoglobulins and TCR, which are specialized for antigen recognition, are the only known members of this family with variable regions that undergo somatic diversification. The function of molecules of the superfamily in adhesion evolutionarily predates specialization for antigen recognition, which occurs only in vertebrates; immunoglobulin superfamily members are present in insects as nervous system adhesion molecules involved in axon guidance and fasciculation¹³⁸. The immunoglobulin domain may have diversified and been adopted so widely in evolution because its stable disulphide-bonded β -strand structure is analogous to a car chassis on which many different styles of bodies and bumpers may be hung. These latter may be analogous to the loops connecting the β -strands, and also to the alternating residues in the β -strands that point outward away from the interior of the domain. An interesting feature of adhesion molecule structure is that, in contrast to immunoglobulins and MHC molecules, which have paired immunoglobulin domains, the domains of ICAM-1 (ref. 130) and NCAM (ref. 139) are unpaired. Immunoglobulin domains studied by X-ray crystallography are ellipsoids with a dimension of 4 nm parallel to the β -strands and 2.5 nm in the two perpendicular dimensions^{17,137}. Electron micrographs of ICAM-1 show that it is a bent rod 18.7 nm long. This is only compatible with a model in which its five domains are unpaired, and arranged end-to-end at a slight angle to the β -strands.



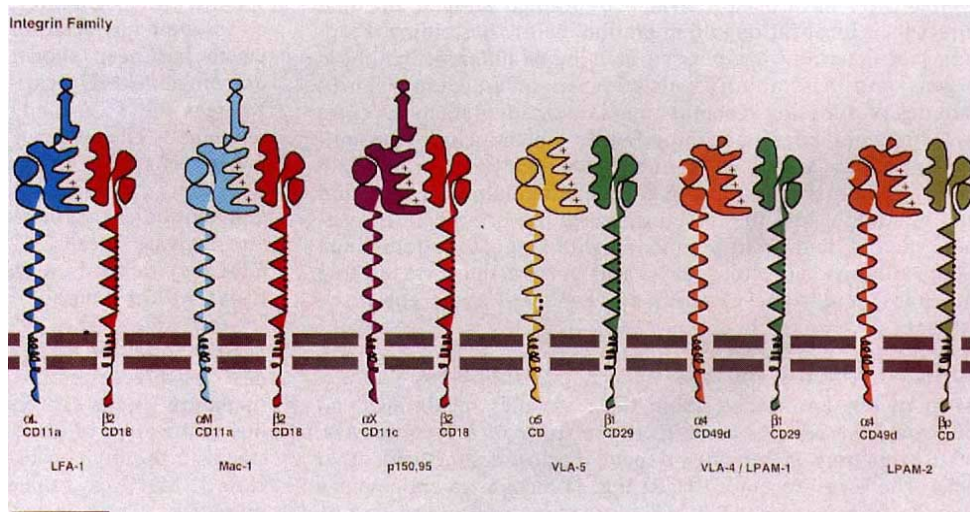
Overall size and shape of the immunoglobulin domains are shown to scale (bar, 10 nm), although schematized, see Fig. 8 for a more accurate scale drawing; dots denote disulphide-bonded cysteines. Dimensions are based on the following information: MHC class I molecule, X-ray crystallography¹⁷; MHC class II, approximation as MHC class I; TCR, approximation of $\alpha\beta$ heterodimer as an Fab fragment¹⁴⁰; ICAM-1, electron microscopy¹³⁰; ICAM-2 (ref. 29), CD2, LFA-3 (ref. 136), CD4 (ref. 13) and VCAM-1 (ref. 70) approximation to two, four or six unpaired domains as in ICAM-1; CD8, a disulphide-linked α - α or α - β dimer of one immunoglobulin domain, the length of the connecting or hinge-like peptide¹³ of 40 or 65 amino acids for α or β , respectively, is arbitrary and is consistent with a spacing of 1.5–3.3 Å per residue¹⁴¹; note that the distance the connecting peptide is shown to extend in CD8 is similar to that which would be predicted for three of the four immunoglobulin domains in CD4, allowing the N-terminal domain of CD4 to occupy a position similar to that shown for the CD8 domain.

interaction of T cells with their targets has been focused principally on killer T-cell interactions, largely because of the simplicity of the killing assay^{5,19–21}. Thus, three molecules have been identified by screening monoclonal antibodies for their ability to inhibit killing and were operationally termed lymphocyte function-related antigens: LFA-1, LFA-2 (CD2) and LFA-3 (Fig. 4a, b). These molecules are now known to account for the antigen-independent adhesion that is induced by prolonged antigenic stimulation of T cells *in vitro*^{7,8,19,22,23} and presumably help localize activated T cells to sites of antigen accumulation in the lymph nodes *in vivo*²⁴.

LFA-1, which is a member of the integrin family (Fig. 2), is expressed on T lymphocytes; its counter-receptor on the target

cell is ICAM-1 or ICAM-2 (for intercellular cell adhesion molecule), both members of the immunoglobulin family (Fig. 1)^{19,21,23,25–29}. LFA-2 or CD2, another member of the immunoglobulin family, is expressed on the T lymphocyte: its counter-receptor on the target cell, LFA-3, is also a member of the immunoglobulin family^{5,19,30,31} (Fig. 1). The LFA-1/ICAM and CD2/LFA-3 interactions can be shown by the effects on adhesion of monoclonal antibodies against the individual molecules to be independent; monoclonal antibodies to any of them, or to the TCR or CD8, can inhibit T-lymphocyte killing, showing that it is a highly complex process requiring cooperation between several different surface molecules^{5,20,21,32}. Indeed, we shall see later that binding of the T-cell receptor can modulate

FIG. 2 Representative integrin family adhesion receptors. Integrins contain α - and β -subunits of approximately 1,100 and 750 amino acids, respectively, which are noncovalently associated. The α -subunits are 25–65% identical in amino-acid sequence and the β -subunits are 37–45% identical; the structural and functional similarities are so strong that integrins should be considered a protein family rather than a superfamily^{21,65}. Although schematic, the overall size and shape of the integrins is shown to scale, based on electron microscopy of VLA-5 (ref. 142) and CD41/CD61 (ref. 143) (bar, 10 nm); the I domain is shown sticking out for emphasis but it may fold up with the rest of the globular head. The dots denote cysteine-rich regions in the β -subunit and a disulphide bridging a cleavage site in some α -subunits. Divalent cation binding sites are symbolized by '+'. Structures are cited in refs 21, 67 and Table I.



the affinity of LFA-1 for its counter-receptor. Adhesive interactions between CD2 and LFA-3 are also regulated by activation of T cells, although by a different mechanism.

Regulated adhesion by the CD2/LFA-3 interaction

The interaction between cells bearing CD2 and LFA-3 is finely poised, and is tipped toward adhesion by T-cell activation. This is reflected in 'rosetting' (Fig. 5), in which erythrocytes, which express LFA-3, adhere *in vitro* to activated T cells expressing CD2: resting T cells do not form rosettes with autologous erythrocytes.

The increase in adhesion between CD2 and LFA-3 on T-cell activation may be largely due to the regulation of the negative charge on the T-cell surface, which is mainly due to sialic acid³³. Close cell-cell contact between circulating cells is opposed by the charge repulsion and by the decrease in entropy required for interdigitation of the surface glycocalyx³⁴; these repulsive interactions seem to be reduced in activated T lymphocytes. The effect of activation on the CD2/LFA-3 interaction can be mimicked by the addition of positive charge through chemical derivatization of erythrocytes or removal of negative charge from erythrocytes or T cells by digestion of the glycocalyx^{35,36}. Charge neutralization alters the morphology of the contact between T lymphocytes and erythrocytes, converting small islands of close contact surrounded by larger areas of greater intermembrane distance to a single large area of close contact³⁶. Despite their larger surface area, T-cell blasts and thymocytes have fivefold less sialic acid per cell than resting T cells³⁷ and are less negatively charged³⁸; this may be a primary factor determining whether the CD2/LFA-3 mechanism and other adhesion mechanisms are active or latent. In lymph nodes, the activated antigen-responsive lymphocytes that aggregate in germinal centres are greatly undersialylated, whereas areas containing B and T cells in rapid transit between blood and lymph are normally sialylated³⁹. In the nervous system as well, cell interactions are regulated by sialylation; polysialylation of NCAM antagonizes its ability to promote adhesion⁴⁰. Theoretical calculations⁴¹ confirm that the attractive and repulsive energies in rosetting are of a similar order of magnitude and thus finely balanced, based on the affinity of the CD2/LFA-3 interaction of 10^6 M^{-1} (refs 30, 42, 43), adhesive receptor surface density and charge density¹⁶³.

CD2 may transduce a signal which augments or synergizes with signals from the TCR⁵. Certain pairs of antibodies against CD2, or combination of one such antibody with multimeric LFA-3, can stimulate T cells, but CD2/LFA-3 interaction alone has no effect^{42,44}. Transfection of cells with CD2 and LFA-3 has confirmed early antibody inhibition results¹⁹ showing CD2/LFA-3 interaction can contribute a 4- to 30-fold enhancement of the immune response^{45,46}. The unusually basic, histidine- and proline-rich 120-amino-acid cytoplasmic region of CD2 is required for stimulation by pairs of monoclonal antibodies^{46,47}; in antigen-specific responses, however, truncation of the cytoplasmic domain of CD2 has given ambiguous results, leaving unclear the relative contributions of adhesion and signalling to enhancement of the immune response by CD2/LFA-3 interaction^{45,46}.

There are two isoforms of LFA-3, derived by differential messenger RNA splicing: one is anchored in the membrane by a glycosylphosphatidyl inositol tail, whereas the other has a classical transmembrane hydrophobic segment and a 12-amino-acid cytoplasmic segment⁴⁸⁻⁵⁰ (Fig. 1). Both are fully active in mediating CD2-dependent adhesion and in promoting T-cell function^{42,45,46,51}. The functional significance of the isoforms remains obscure, although it is intriguing that the neural cell adhesion molecule (NCAM) also has anchor isoforms^{40,52}.

ICAM-1 and ICAM-2 counter-receptors for LFA-1

LFA-1, although originally identified by monoclonal antibodies that inhibit T cell-mediated killing, is also required for a broad

range of other leukocyte functions, including T-helper and B-lymphocyte responses, natural killing, antibody-dependent cytotoxicity mediated by monocytes and granulocytes, and adherence of leukocytes to endothelial cells, fibroblasts and epithelial cells^{19,21}. A counter-receptor for LFA-1, ICAM-1, was identified using a simple assay called homotypic adhesion (Fig. 6a), in which homogeneous cell populations such as B- or T-cell lines adhere to one another to form multicellular clusters^{19,53}. Aggregates form in this assay only if the lymphocytes have been stimulated with phorbol esters, and adhesion is completely inhibited by monoclonal antibodies against LFA-1 and is not observed with cell lines established from patients genetically deficient in LFA-1 (see below). LFA-1⁺ cells can however coaggregate with LFA-1⁻ cells⁵⁴; and an LFA-1 counter-receptor, ICAM-1, was defined by immunizing mice with LFA-1⁺ cells, and selecting monoclonal antibodies that would inhibit LFA-1-dependent homotypic adhesion⁵⁵. ICAM-1 is a member of the immunoglobulin superfamily^{27,28}, with five immunoglobulin domains (Fig. 1).

In contrast to LFA-1, which is restricted to leukocytes, ICAM-1 can be expressed on a wide variety of cells and its induction in inflammation is an important means of regulating LFA-1/ICAM-1 interactions^{21,53} and thereby presumably inflammatory responses. In the absence of an inflammatory response, ICAM-1 is expressed on only a few cell types⁵⁶. Its importance has been demonstrated *in vitro* by blocking T-cell killing with antibody to ICAM-1 (ref. 57), and by transfection experiments in which fibroblasts expressing suboptimal levels of MHC molecules can be enabled to activate T-helper cells by cotransfection with the gene encoding ICAM-1⁵⁸. This is consistent with its natural expression during the response to a specific antigen. ICAM-1 is expressed both on the follicular dendritic cells and on the

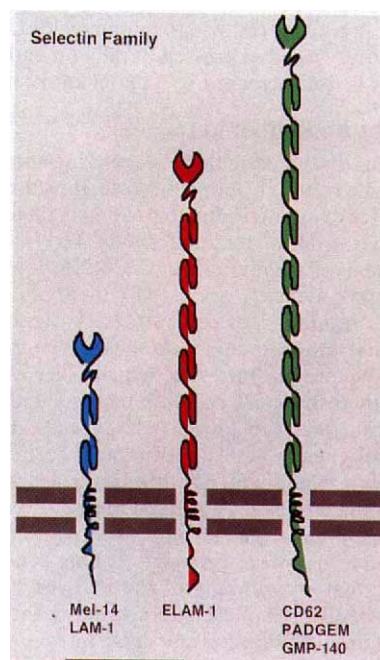


FIG. 3 Selectins. Selectins have an N-terminal domain of 117–120 amino acids which is homologous to a variety of Ca^{2+} -dependent animal lectins¹⁴⁴ including hepatic galactose receptors, soluble mannose-binding lectins, and invertebrate lectins, as well as to proteins known to bind ligands independently of carbohydrate, including the low-affinity receptor for IgE (CD23). After the N-terminal lectin domain is an EGF (epidermal growth factor) motif of 34–40 amino acids, and then short consensus repeat motifs of 62 amino acids, as found in many proteins involved in regulating complement activation. Structures are from refs 110, 112–115. Selectin domains are shown roughly to scale (bar, 10 nm); the short consensus repeats (SCR) extend 4.1 nm each, based on a length of 33 nm (ref. 145) for 8 SCR (ref. 146); EGF repeats extend about 2.3 nm (ref. 147), and the lectin-like N-terminal domain is modelled as a globular sphere¹⁴⁴.

activated B lymphocytes found in germinal centres⁵⁶, and may contribute through homotypic aggregation to formation of germinal centres.

Inflammatory mediators, including lipopolysaccharide, γ -interferon (γ -IFN), interleukin-1 (IL-1) and tumour necrosis factor (TNF) cause strong induction of ICAM-1 in a wide variety of tissues and greatly increase binding of lymphocytes and monocytes through their cell surface LFA-1 (refs 19, 21, 53, 59). Endothelial, fibroblastic and epithelial cells vary as to which cytokines are capable of inducing ICAM-1 expression, and the types of mediators released may therefore help regulate differing patterns of cell localization induced by inflammatory stimuli. Binding of leukocytes to endothelium is the first step in localization of circulating cells at an inflammatory site (Fig. 7a). *In vivo*, ICAM-1 induction accompanies T cell-mediated hypersensitivity reactions in the skin⁶⁰, and, after administration of γ -IFN and IL-1, appearance of ICAM-1 on endothelial cells correlates with sites of mononuclear cell infiltration⁶¹. ICAM-1 induction is largely regulated at the mRNA level^{27,28}. Increased surface expression is first seen after 4 h, and is usually maximal by 24 h (ref. 53).

A second LFA-1 ligand, differing in tissue distribution from ICAM-1, was originally defined by the ability of antibodies against LFA-1, but not those against ICAM-1, to inhibit certain cell adhesion assays. On this basis, an ICAM-2 complementary DNA was isolated from an expression library by screening for binding of transfected cells, in the presence of monoclonal antibody against ICAM-1, to purified LFA-1 coated on Petri dishes²⁹. ICAM-2 has two immunoglobulin-like domains, in contrast to ICAM-1 which has five (Fig. 1), and these are 35% identical to the two N-terminal domains of ICAM-1. ICAM-1 and ICAM-2 are much more similar to one another than to other members of the immunoglobulin superfamily, and thus represent a subfamily specialized to interact with LFA-1. Unlike ICAM-1, ICAM-2 is well expressed basally on endothelial cells and its mRNA is not increased by inflammatory mediators.

LFA-1 avidity and adhesion

The mechanisms discussed so far for regulating adhesive interactions operate on a relatively long timescale. Increased expression of ICAM-1 after cytokine induction is detectable *in vitro* or *in vivo* after 4–6 h, and is maximal by 9–24 h^{21,53,61}. This time course is typical of regulation at the RNA level of surface adhesion receptor density, and seems to apply to CD2 and LFA-3 as well. Alteration of cell surface charge by changes in glycoprotein sialylation requires *de novo* glycoprotein biosynthesis⁶² and glycoprotein turnover, which takes about 12–24 h. Yet adhesion by cytotoxic T cells can be regulated over a much shorter time scale; they can adhere to target cells, deliver a lethal hit, detach and engage with another target cell, with a cycle time as short as 1–5 min (ref. 63). Moreover, whereas cytotoxic cells stimulated *in vitro* show a general increase in adhesiveness, cells stimulated *in vivo* adhere only to those cells bearing the antigen to which they were primed^{19,20}. This can be explained by the finding that crosslinking of the TCR on resting T lymphocytes transiently stimulates adhesiveness through LFA-1, allowing regulation of adhesion and detachment over a timescale of minutes²³.

This rapid modulation of adhesion is due to qualitative rather than quantitative changes in the cell-surface expression of adhesive molecules. Homotypic adhesion of leukocytes (Fig. 6a), which is dependent on LFA-1 and ICAM-1, is stimulated by treatment for 1 h with phorbol ester but is accompanied by no increase in LFA-1 or ICAM-1 surface expression^{54,55}. By testing the binding of cells expressing LFA-1 and ICAM to plastic substrates coated with either purified ICAM-1 or purified LFA-1 (Fig. 6b), however, it can be shown that stimulating resting T lymphocytes with phorbol esters or crosslinking the TCR with monoclonal antibodies converts cellular LFA-1 from a low- to a high-avidity state, with no change in surface density²³. Cellular

ICAM, by contrast, is constitutively avid. These changes influence the ability of stimulated peripheral blood lymphocytes to form conjugates with target cells. Conjugate formation is inhibited completely by LFA-1 and only marginally by CD2 monoclonal antibodies, showing that LFA-1 is primarily responsible for regulating the avidity of cell-cell interactions. Avidity peaks 5–10 min after stimulation of the TCR and returns to resting values by 30 min. The transience of the high-avidity state provides a mechanism for regulating lymphocyte deadhesion.

Thus, in the absence of antigen, the equilibrium governing adherence favours free, mobile T lymphocytes and efficient immune surveillance. Contact of TCRs with cells bearing specific antigen generates intracellular signals that lead to the conversion of LFA-1 to a high-avidity state and regulates LFA-1/ICAM-1-dependent adhesion in an antigen-specific manner. LFA-1 is an adhesion servomotor controlled by the TCR.

The integrin family

LFA-1 is a member of the integrin family (Table 1), perhaps the most versatile of the adhesion molecule families. Each integrin molecule comprises an α - and a β -subunit (Fig. 2) and three subfamilies of integrins can be distinguished by their β subunits: these are known as the $\beta 1$ (CD29), $\beta 2$ (CD18) and $\beta 3$ (CD61) integrins.

LFA-1 belongs to the $\beta 2$ subfamily and is most closely related to two other integrins, Mac-1 and p150,95 with which it shares the $\beta 2$ subunit²⁵ (Fig. 2). These three $\beta 2$ integrins are also known as the leukocyte integrins because their expression is limited to white blood cells. Mac-1 and p150,95 are particularly important in adhesion of myeloid cells to other cells and to ligands that become insolubilized during activation of the complement and clotting cascades²¹ (Table 1). The importance of the leukocyte integrins is illustrated in congenital leukocyte adhesion deficiency (LAD) in which they are deficient because of mutations in the common $\beta 2$ subunit^{21,64}. Patients have recurring infections, often fatal in childhood unless they are corrected by bone marrow transplantation. Neutrophils from these patients fail to orient and migrate in response to chemoattractants and are unable to bind to and cross the endothelium at sites of infection, so that pus fails to form. This is a most striking example of the role of adhesion molecules in leukocyte localization *in vivo*.

The $\beta 1$ integrin subfamily includes receptors that bind to the extracellular matrix components fibronectin, laminin and collagen (Table 1) and they are expressed on many non-haematopoietic and leukocyte cell types. These receptors are likely to play a general part in tissue organization by binding to molecules in the extracellular matrix within many tissues and in the basement membranes found in muscle, the nervous system,

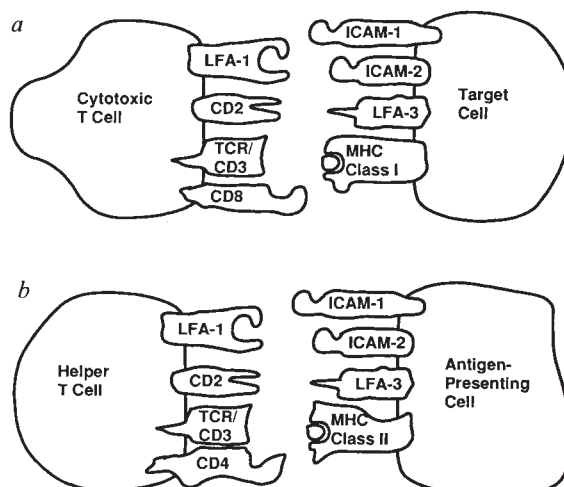


FIG. 4 Antigen-specific T-cell interactions.

TABLE 1 Integrin family of cell-cell and cell-matrix receptors

Subunits	Names	α -subunit*		Ligands†	RGD role	Distribution		Ref.‡
		I	C			Non-leukocyte‡	Leukocyte§	
α L β 2	CD11a/CD18, LFA-1	+	—	ICAM-1,2	—		B, T, M, G	21
α M β 2	CD11b/CD18, Mac-1, CR3	+	—	C3bi, FX, FB	+?		M, G	21
α X β 2	CD11c/CD18, p150, 95	+	—	?	?		M, G	21
α 1 β 1	CD-/CD29, VLA-1	?	—	LM, CO	—	F, BM	B', T'	67
α 2 β 1	CD49b/CD29, VLA-2, gplalla, ECMRII	+	—	LM, CO	—	P, F, EN, EP	T'	154-157
α 3 β 1	CD-/CD29, VLA-3, ECMRI	—	+	FN, LM, CO	—	EP, F		155
α 4 β 1	CD49d/CD29, VLA-4, LPAM-1	—	!	FN, VCAM-1	—	NC, F	B, T, M, LGL	68, 69, 71, 74, 76
α 5 β 1	CD-/CD29, VLA-5, FNR, gp1c/IIa, ECMRVI	—	+	FN	+	F, EP, EN, P	Th, T	155
α 6 β 1	CD49f/CD29, VLA-6, gp1c/IIa	—	+	LM	—	P	T	94, 158
α 4 β p	CD49d/CD-, LPAM-2	—	!	?	?		T	82
α 6 β 4	CD49f/CD-, α E β 4	—	+	?	—	E		77-79
α Ib β 3	CD41/CD61, gpIIbIIIa	—	+	FB, FN, vWF, FB	+	P		95
α V β 3	CD51/CD61, VNR	—	+	VN, FB, vWF, TSP	+	EN	B', M	95, 159, 160
α V β 1	CD51/CD29	—	+	FN	+	NC, F		161
α V β 5	CD51/CD-, α V β 5	—	+	VN, FN	+	C, F, EP	M	80, 81, 160

*I, I domain; C +, cleavage to disulphide-linked heavy and light chains at amino acid 853-860; !, cleavage to nondisulphide-linked chains at roughly amino acid 573.

† LM, laminin; CO, collagen; FN, fibronectin; FB, fibrinogen; FX, factor X; VN, vitronectin; vWF, von Willebrand factor; TS, thrombospondin.

‡ EN, endothelial cells; EP, epithelial cells; F, fibroblasts or other connective tissue; NC, neural crest, melanocytes; P, platelets; C, carcinomas; BM, basement membrane-associated.

§ B, B lymphocytes; T, T lymphocytes; ', activated lymphocytes only; Th, thymocytes; M, monocytes; G, granulocytes; LGL, large granular lymphocytes. Some data from Leukocyte Typing Database III (ref. 162) and IV.

|| General refs 2, 21, 65-67.

and underlying the epithelium and endothelium^{2,65,66}. The β 1 family molecules have been designated VLA (very late activation) because two of them, VLA-1 and VLA-2, appear on lymphocytes 2-4 weeks after antigen stimulation *in vitro*⁶⁷. In fact, some VLA molecules are basally expressed on leukocytes, and their expression on nonhaematopoietic cells does not require activation (Table 1). Induction of VLA-1, -2, -3 and -5 expression after leukocytes cross the endothelial barrier may be of great importance in controlling leukocyte localization in inflammation.

VLA-4 (CD49d/CD29) is an unusual β 1 integrin expressed on resting lymphocytes, monocytes and neural crest-derived cells, and functions as both a matrix and cell receptor⁶⁷. As a matrix receptor, it binds to an alternatively spliced domain of fibronectin distinct from the classical cell binding site recognized by VLA-5^{68,69}. As a cell receptor, it binds to a molecule recently described as VCAM-1 or INCAM110 that is a member of the immunoglobulin superfamily⁷⁰⁻⁷². This molecule is induced by inflammatory mediators on endothelium with kinetics similar to ICAM-1 and its interaction with VLA-4 provides an explanation for earlier evidence of a second lymphocyte-endothelium adhesion mechanism distinct from the LFA-1/ICAM interaction^{59,73} (Fig. 7b). In congenital deficiency of the β 2 integrins, which does not affect VLA-4, lymphocytes retain the ability to migrate across the endothelium at inflammatory sites⁶⁴. This seems related to expression of VLA-4 by lymphocytes and not by neutrophils (Fig. 7c). Involvement of VLA-4 in T cell-mediated killing⁷⁴ and in homotypic adhesion⁷⁵ suggests some functional redundancy with LFA-1. VLA-4 also helps mediate lymphocyte recirculation⁷⁶, as described below.

The complexity of the integrin family has recently been increased by the discovery of novel β -subunits that can associate with the α 4, α 6 and α V subunits alternatively to the previously described β 1 and β 3 subunits⁷⁷⁻⁸² (Table 2). Both α - and β -subunits affect ligand specificity. Their combinatorial use confers considerable diversity in ligand recognition, and differences in transmembrane and cytoplasmic domains may also help regulate communication between the inside and outside of the cell.

Structure and regulation of integrins

The structural domains of integrins (Fig. 2) have been correlated with ligand binding by crosslinking to peptides containing the sequence Arg-Gly-Asp (RGD in the single-letter amino-acid code), a ligand recognition motif for several but not all integrins (Table 1). On the β 3 subunit, ligand peptides are crosslinked within residues 109-171 (ref. 83). This is the most highly conserved region among the β 1, β 2 and β 3 subunits, and in LAD

single amino-acid substitutions in this region of β 2 prevent association with the α -subunit (ref. 84); thus close association of the α -subunit with this region of the β -subunit may form a ligand-binding pocket. Integrin α -subunits have three or four tandem repeats of a putative divalent cation-binding motif (Fig. 2), and require Ca^{2+} or Mg^{2+} for function²¹. LFA-1 α -subunit has three such repeats and binds Mg^{2+} ; this correlates with the requirement for Mg^{2+} in T-cell adhesion and in binding of purified LFA-1 to purified ICAM-1 (ref. 23). The divalent metal-binding motif in integrins has only five of six predicted metal coordination sites. It has been proposed that in integrin binding to RGD-containing ligands, the Asp residue (D) in RGD binds to the metal held in the divalent cation-binding pocket of the α -subunit, forming a sixth coordination site⁸⁵. Consistent with this, a ligand is crosslinked to amino acids 294-314 of the α -subunit of α Ib β 3, which define the second divalent cation-binding site⁸⁶.

Single integrin domains may be involved in ligand binding. All three leukocyte integrin α -subunits and the VLA α 2 subunit have a domain of 200 amino acids not present in other integrin α -subunits, and hence termed the 'inserted' or I domain. The I domains are homologous to ligand-binding repeats in von Willebrand factor and other proteins, and may confer modes of ligand recognition in addition to those shared by all integrins²¹. Cysteines are notably few in the putative ligand binding regions of the α - and β -subunits, permitting conformational changes that regulate ligand binding.

Interactions of integrins with the cytoskeleton may be regulated by binding to ligands, and conversely, may help regulate ligand binding, thus mediating a bidirectional dialogue across the membrane. Several of the integrins can localize near to focal contacts, areas where the cell membrane is closely opposed to the extracellular matrix substrate and where actin bundles terminate, surrounded by a ring of vinculin and talin⁸⁷. Talin seems to interact with the cytoplasmic domain of α 5 β 1 (ref. 88). Talin redistributes with LFA-1 to sites of antigen-specific adhesion and cocaps with LFA-1 after stimulation with phorbol ester¹⁴; talin association may be a widespread feature of integrins. It is intriguing that redistribution of LFA-1 and talin is highly sensitive to low antigen concentrations and may correlate with the high-avidity state of LFA-1⁸⁹.

Like many receptors, integrins transduce information from the outside to the inside of the cell. The growth and differentiation of many connective tissue and nervous system cells is affected by their substrates, largely through integrins^{2,65,66}. Examples in the immune system include regulation of T-cell proliferation by LFA-1 (refs 90-92), VLA-5 (ref. 93), VLA-4 and VLA-6 (ref. 92).

Integrins are novel receptors with respect to the type of inside-out signalling described for LFA-1, in which signals from the cytosol are transduced across the membrane to generate changes in extracellular functions such as adhesion. The avidity of other integrins besides LFA-1 seems to be regulated. On lymphocytes, the avidity of the $\beta 1$ integrins VLA-4 and VLA-5 for fibronectin and VLA-6 for laminin is increased analogously to LFA-1 after TCR crosslinking⁹⁴. On unactivated platelets, the integrin gpIIb/IIIa does not bind fibrinogen, but on activation binds soluble fibrinogen with a dissociation constant K_d of 29–45 nM (ref. 95). The high-avidity state of gpIIb/IIIa seems to be permanent rather than transient. The mechanism of avidity regulation is unclear, but the ability of antibodies to detect conformational changes in LFA-1 (ref. 96) and in gpIIb/IIIa (ref. 95) in sites distinct from the ligand-binding site suggests conformational changes in the ligand binding site may also occur. On stimulation with chemoattractants, neutrophils transiently adhere to other neutrophils, endothelial cells and to C3bi-coated cells and there is some evidence that this may be due to a transient change in avidity of Mac-1 (refs 97–99). The cytoplasmic domains of integrins, perhaps by interaction with other cellular proteins, may signal the changes in the extracellular domains that regulate avidity. The LFA-1 β -subunit (integrin $\beta 2$) cytoplasmic domain is required for functional activity in binding to ICAM-1, as shown by transfection of patient cell lines that genetically lack the β -subunit with intact and truncated β -subunit cDNAs (ref. 100, and M. L. Hibbs, H. Xu, S. A. Stacker and T.A.S., unpublished data).

Lymphocyte education

Lymphocytes newly emigrated from the thymus are considered 'naïve', and remain so until they encounter and are stimulated by specific antigen. They then become longer-lived 'memory' lymphocytes. As discussed above, transient alterations in adhesion mechanisms lasting for minutes to days accompany lymphocyte activation. But permanent alterations in surface density also occur, as a result of the transition from the naïve to memory phenotype. Shortly after antigen stimulation, naïve T lymphocytes of both CD4⁺ and CD8⁺ subsets acquire increased levels of a cohort of surface molecules including the adhesion receptors CD2, LFA-1 and VLA family members^{94,101,102} (Table 2). Increased expression of these surface molecules persists after the stimulated lymphocytes have reverted to the resting state, probably lasting for the life of the memory cell. The changes in surface phenotype of memory T cells may have important consequences for their localization because they occupy distinct microenvironments within lymphoid organs¹⁰³ and have different recirculation routes¹⁰⁴.

TABLE 2 Conversion of naïve to memory T lymphocytes alters surface molecule phenotype*

Molecule	Difference in expression (-fold increase)
CD2	2.8
LFA-1	2.4
LFA-3	– to +
CD49d (VLA-4)	2.7
(VLA-5)	3.6
CD49f (VLA-6)	3.4
CD29 (VLA- β , 4B4)	3.7
CD44 (Hermes Pgp-1)	2.1
CD45RO (UCHL1)	29
CD45 RA (2H4)	+ to –
CD4	1.0
CD8	1.0
TCR (CD3)	1.0

* Modified from refs 94, 102.

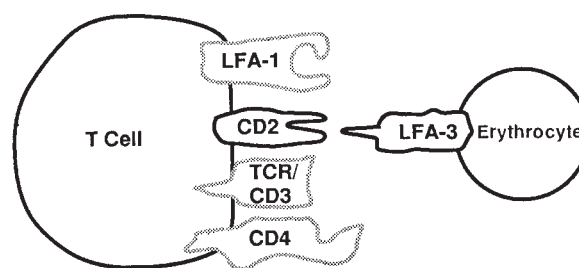


FIG. 5 T-cell rosetting with erythrocytes.

Naïve and memory T-cell subsets differ in lymphokine secretion, in some functional assays^{101,102,105,106}, and in other important respects. Memory T cells seem to be more sensitive to antigen, because they are responsive to stimulation by much lower concentrations of monoclonal antibody against TCR, although they have quantities of TCR, CD8 and CD4 identical to naïve T cells¹⁰⁷. Increased expression of the LFA-1 and CD2 molecules should also enhance their sensitivity to antigen by facilitating interactions with antigen-presenting cells.

Lymphocyte recirculation receptors

Patrolling the body in search of foreign antigen, lymphocytes leave the blood, migrate through lymphoid organs and other tissues, and enter the lymphatics, whence they return to the blood through the thoracic duct. The peripheral lymph nodes draining the skin and the Peyer's patch and gut-associated lymph nodes draining mucosal surfaces differ in the types of antigens to which lymphocytes are exposed. Lymphocytes from adult animals differ from those in newborns in that, when collected from specific lymph nodes, they show a twofold preference for recirculation to lymph nodes of the type from which they came^{24,108}. This suggests that priming by specific antigen may alter surface phenotype to enable selective recirculation to the type of secondary lymphoid organ where specific antigen was first encountered.

Lymphocytes in the blood enter lymph nodes by binding to specialized 'high' endothelial cells. Lymphocyte suspensions overlaid on sections of lymph nodes bind to these specialized venules, and 'recirculation' or 'homing' receptors on lymphocytes have been defined by monoclonal antibodies that block binding to the high endothelial cells of specific types of lymph nodes^{24,108,109}. Molecules, termed 'addressins', selectively expressed on specialized high endothelium in different types of lymph nodes, are good candidates for the homing receptor ligands^{24,108,109}. Binding and immigration to lymph nodes may be a cooperative process involving several receptors on the lymphocyte and counter-receptors on the endothelium, analogous to antigen-specific interactions (Fig. 4) because CD44, LFA-1, VLA-4 and Mel-14/LAM-1 on the lymphocyte have all been implicated in binding to high endothelial venules (HEV). The more interesting candidates for specific receptors are Mel-14/LAM-1 as a peripheral lymph node receptor, and the VLA-4 α -subunit associated with either of two integrin β -subunits as a Peyer's patch receptor^{24,82,108–110}. But the function of these molecules in adhesion hardly seems limited to lymphocyte recirculation, as discussed above for VLA-4.

Alternatively to emigration through HEV, lymphocytes may emigrate through endothelium within a tissue such as skin, and enter a lymph node through the afferent lymph. Memory T lymphocytes almost exclusively emigrate from blood through tissue endothelium, whereas naïve lymphocytes emigrate through HEV (ref. 104). It is thus possible that the endothelia of skin and mucosa may differ in expression of ligands that enable selective recirculation through these tissues. Congruent with this idea, the candidate homing receptors CD44 and VLA-4 show increased expression on the memory T-lymphocyte subset (Table 2) and LAM-1 (Leu8/TQ1) is preferentially expressed

on a distinctive but overlapping memory lymphocyte subset¹¹¹. Differences of several fold in surface density of these receptors may be adequate to give rise to selectivity in recirculation.

The role of selectins

The Mel-14/LAM-1 molecule is a representative of a novel class of molecules termed 'selectins', with an N-terminal lectin domain and diverse roles in adhesion^{109,110,112-115} (Fig. 3). The finding of the lectin-like domain in Mel-14/LAM-1 correlates with the Ca^{2+} requirement for lymphocyte binding to peripheral lymph node endothelium and evidence that the counter-receptor is carbohydrate-like^{108-110,113}. The number of short consensus repeats, which varies from two to nine in the three different selectins discovered so far, may serve to position their N-terminal lectin-like putative binding sites at varying distances from the plasma membrane (Fig. 3).

All three selectins help regulate leukocyte binding to endothelium at inflammatory sites (Fig. 7c). The selectin Mel-14 not only functions as a lymphocyte recirculation receptor, but also contributes to neutrophil emigration at inflammatory sites^{24,116}. After stimulation of neutrophils with chemoattractants, Mel-14/LAM-1 is rapidly shed from the cell surface¹¹⁷. The endothelial leukocyte adhesion molecule, ELAM-1, is a selectin that is transiently expressed on endothelial cells 2-8 h after stimulation with IL-1 and other inflammatory agents, and mediates a neutrophil adhesion pathway distinct from that mediated by ICAMs and leukocyte integrins^{112,118}. The neutrophil chemoattractant IL-8, which is secreted by activated endothelial cells, acts on neutrophils to inhibit binding to ELAM-1 (ref. 119). The proteolytic release of Mel-14 from the cell surface upon neutrophil activation, and the similar inactivation of the ELAM-1 counter-structure on the neutrophil, suggests that Mel-14 and ELAM-1 function in an early step in neutrophil binding to the endothelium, before transendothelial migration. This contrasts with integrins, which are increased on the neutrophil surface by mobilization from granule compartments within minutes after stimulation of neutrophils with chemoattractants, and then remain permanently upregulated^{64,117}. Although both selectins and integrins can regulate neutrophil adhesion to endothelium, when selectins mediate adhesion, integrins are still required for the subsequent event of transendothelial migration^{120,121} (Fig. 7a). This may explain why congenital deficiency of the leukocyte integrins so completely inhibits neutrophil emigration from the blood⁶⁴.

A third selectin called PADGEM, GMP-140 or CD62 is stored in α -granules of platelets and Weibel-Palade bodies of endothelial cells, and is rapidly mobilized to the surface of these cells after stimulation by products of the clotting cascade such as thrombin, where it mediates adhesion of neutrophils and monocytes^{115,122}. Thus, selectins function in a wide range of cell interactions in the vasculature and are expressed both on leukocytes and endothelial cells.

Virus receptors

Several cell adhesion receptors are subverted as virus receptors¹²³. The human immunodeficiency virus (HIV), which causes AIDS, binds to CD4. Although rhinoviruses, which cause common colds, have evolved more than 100 non-crossreactive antigenic variants in an attempt to evade the immune response, 90% of them bind to the same receptor, ICAM-1.

The use of cell-adhesion receptors by viruses may be more than coincidental. First, virus-cell and cell-cell adhesion are in principle very similar. Both require accessible cognate sites on the receptor, both involve multivalent interactions and both may be facilitated by receptor redistribution to the site of adhesion. Second, binding to molecules that participate in the immune response has important consequences for the host-virus relationship. Human immunodeficiency virus (HIV) infects and kills or renders anergic CD4^+ T-helper cells, and downregulates CD4 expression¹²⁴. Binding of HIV or its shed surface glycoprotein

gp120 may transduce signals through CD4 which interfere with normal responsiveness¹²⁵. HIV can spread by fusion of infected cells with uninfected cells to form syncytia. This requires CD4 and gp120 but also LFA-1 (ref. 126). Rhinoviruses, by contrast, rather than thwarting the immune response, use it to their own ends. Mucous secretions and sneezing induced by the immune response facilitate infection of other individuals. If ICAM-1 has a signal transducing function on antigen-presenting cells, this may be mimicked by the virus and may serve its ends by stimulating production of cytokines which increase nasal secretions carrying the virus.

In view of the rationales discussed above, it is of considerable interest that viruses have evolved to bind to the same regions of CD4 and ICAM-1 as do their cell adhesion counter-structures. HIV binds to the first and part of the second immunoglobulin-like domains of CD4 (refs 127-129); the same overall region of CD4 binds to MHC class II molecules¹²⁹. LFA-1 and rhinoviruses bind to overlapping but distinct regions of the first immunoglobulin-like domain of ICAM-1 (ref. 130). The binding site for ICAM-1 is hypothesized to be in a 'canyon' on the rhinovirus surface which is too narrow to admit an antibody¹⁶⁴, consisting of paired immunoglobulin domains, but is of the right dimensions to fit unpaired domains as exist in ICAM-1 (ref. 130).

Close encounters at the membrane

We are just beginning to understand adhesion on a molecular scale (Fig. 8). The size and shape of several immune system adhesion molecules are known by electron microscopy or X-ray crystallography, and others may be modelled from the structure of homologous molecules or constituent domains (Figs 1, 2 and 3 legends). The binding sites for several of the adhesion receptors and their counter-receptors are known (Fig. 8 legend), allowing prediction of how close together two cell membranes, or a cell membrane and the extracellular matrix, would have to come to allow interaction. A model of the distances over which adhesion receptors would mediate cellular interactions can then be constructed (Fig. 8). This model can be compared with measurements of distances made in micrographic studies^{131,165}. Furthermore, the sizes of two major components of the leukocyte glycocalyx that bear most of the cell-surface sialic acid¹³², CD43

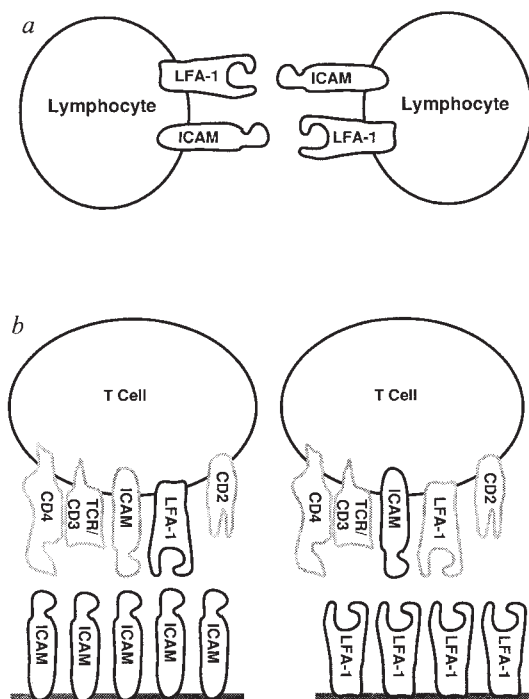
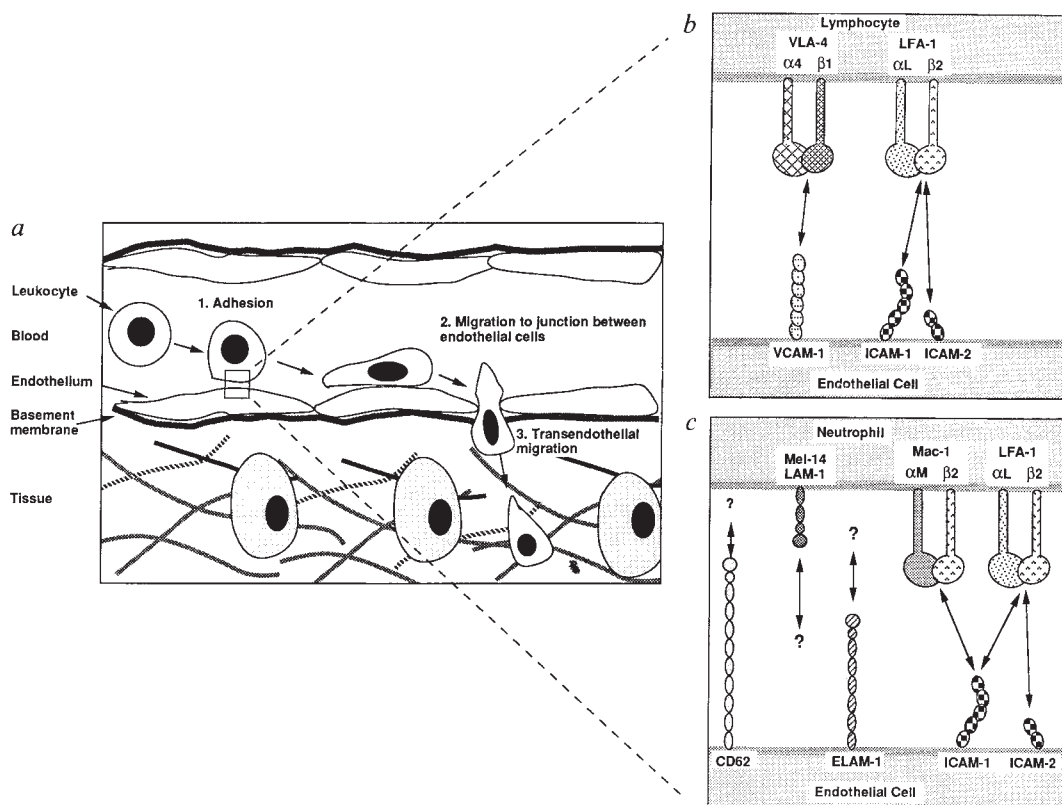


FIG. 6 Homotypic adhesion (a) and adhesion to purified molecules in artificial membranes (b).

FIG. 7 Leukocyte extravasation (a), and mechanisms for adhesion of lymphocytes (b) and neutrophils (c) to endothelium.



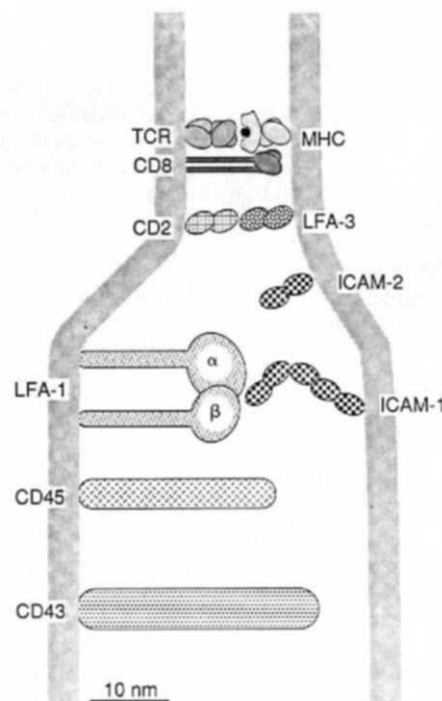
and CD45, are included in Fig. 8 as a reminder of the scale of the glycocalyx, which will oppose cell-cell adhesion due to negative-charge repulsion and the loss of entropy involved in compressing or interdigitating the glycocalyxes of two contacting cells³⁴.

The model (Fig. 8) predicts two classes of adhesion receptor interactions that differ significantly in the distance between the plasma membranes of the two closely apposed cells. The TCR-MHC and CD2/LFA-3 interactions are predicted to occur within an intermembrane distance of ~13 nm or less. This is compatible with electron microscopic measurements of intermembrane

distances of 7.5 nm for point contacts of microvilli (0.2-μm wide) and 17 nm for broad areas several μm wide of membrane apposition in interactions of mitogen-stimulated T cells with target cells¹⁶⁵.

By contrast, the interactions of LFA-1 with ICAM-2 and ICAM-1 are predicted to occur within an intermembrane distance roughly equal to or less than 27 or 36 nm, respectively (Fig. 8). There may be important qualitative differences between these two types of membrane contacts, and they could occur in different membrane domains within the zone of adhesion between two cells. Very close contact mediated by the TCR-

FIG. 8 Scale models of interacting adhesion receptors. Immunoglobulin superfamily members and integrins are drawn to scale (Figs 1, 2 legends). Glycocalyx components: CD43, the 235-residue extracellular domain^{148,149} with one in five O-linked amino acids¹³² is modelled as 235/420 the length of glycosialin (CD42b), which has 420 amino acids with a similar O-linked carbohydrate content^{150,151}; CD45 is based on electron micrographs¹⁵². The lipid bilayer is drawn to scale as 4–5-nm thick; protein transmembrane and cytoplasmic domains are omitted. The presence of hinge or connecting regions between globular domains and the membrane-spanning domain introduces uncertainty into how far some of these molecules extend from the membrane, because an extended polypeptide would extend much farther than globular regions with the same number of amino acids. But connecting regions are absent or short except for CD8, and are already included in the lengths determined by electron microscopy for integrins and CD45. Carbohydrate side chains are not shown; the sizes of molecules based on electron microscopy would take into account both protein and carbohydrate. The MHC class I peptide-binding site is assumed to interact with the complementarity-determining region of the TCR¹⁴⁰. The binding site in MHC class I for CD8 is in the membrane proximal α3 domain^{15,16}; the binding site for MHC is assumed to be in the CD8 immunoglobulin domain, analogously to the MHC binding site in CD4 (ref. 129). ICAM-1 binds to LFA-1 through its N-terminal immunoglobulin domain¹³⁰; the binding site in ICAM-2 is modelled in its homologous N-terminal domain. The ICAM binding site in LFA-1 is assumed to be in the membrane-distal globular domain of integrins as predicted¹⁴²; the exact location within this domain will not affect overall conclusions. The binding site for LFA-3 in CD2 is in the N-terminal immunoglobulin domain¹⁵³, and the binding site in LFA-3 is assumed to be in a homologous position.



MHC and CD2/LFA-3 interaction would be expected to require extensive interdigitation of the membrane glycolyxes of the two adhering cells, hinder the lateral mobility of surface glycoproteins, and either exclude major glycolyx components such as CD43 and CD45 from the zone altogether or require that they assume a less extended conformation or change their orientation to the membrane. Because the cytoplasmic domain of CD45 is a tyrosine phosphatase that acts on the tyrosine kinase associated with CD4 and CD8 (ref. 133), such considerations could affect signalling.

The longer range contacts mediated by the LFA-1 interaction with ICAM-1 and ICAM-2 are predicted to involve much less glycolyx interdigitation. This may be compatible with lateral movement of one membrane surface relative to the other, without significant frictional drag imposed by interlocking of the apposed glycolyxes. Migrating cells do not possess focal contacts, which have a cell to substrate distance of 10–15 nm, but instead have close contacts with a cell to substrate distance of ~30 nm (ref. 131), in good agreement with the distance predicted for LFA-1/ICAM interaction, and analogously, for integrin-matrix interaction. There is now good evidence that lipids¹³⁴ and membrane proteins¹³⁵ are drawn forward by extension of the leading edge of the cell. Although evidence is best for the dorsal surface, results suggest that the ventral surface of the membrane also translates forward with respect to the substrate. LFA-1 and other integrins could serve as transiently fixed points in the membrane that connect the cytoskeleton to the extracellular environment, with a cell-cell or cell-substrate distance that allows membrane lipid and other proteins on the same cell surface to flow laterally in a tide drawn by membrane extension in another area of the cell. Localized changes in integrin avidity, with high avidity at the leading edge of the cell and low avidity at the trailing edge may help regulate and drive cell migration²³, in conjunction with tension generated by the cytoskeleton. These two mechanisms for regulating cell migration may be coordinated by interactions between the cytoplasmic domains of integrins and the cytoskeleton.

Concluding remarks

I have emphasized the dynamic role of lymphocyte adhesion receptors in regulating lymphocyte antigen-specific interactions, localization in lymphoid and nonlymphoid organs, and in bidirectionally transmitting information which affects cellular differentiation and responsiveness and interaction with the environment. Adhesion receptors modulate interactions on different temporal scales and at different distances from the cell surface. There are important interactions between antigen receptors and adhesion molecules involving signalling pathways and interactions with gene expression. Further studies on three-dimensional structure and interactions with signalling pathways and the cytoskeleton promise to provide exciting insights into the mechanism of function of adhesion receptors. The role of these molecules *in vivo* in guiding cell interactions and localization in the complex microarchitecture of lymphoid organs, as well as in immune responses in other tissues, is another area that promises to yield rich insights. □

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ARTICLES

A 1,400-year tree-ring record of summer temperatures in Fennoscandia

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Tree-ring data have been used to reconstruct the mean summer (April–August) temperature of northern Fennoscandia for each year from AD 500 to the present. Summer temperatures have fluctuated markedly on annual, decadal and century timescales. There is little evidence for the existence of a Medieval Warm Epoch, and the Little Ice Age seems to be confined to the relatively short period between 1570 and 1650. This challenges the popular idea that these events were the major climate excursions of the first millennium, occurring synchronously throughout Europe in all seasons. An analysis of past warming trends suggests that any summer warming induced by greenhouse gases may not be detectable in this region until after 2030.

THE collection of living and remnant Scots pine (*Pinus sylvestris* L.) from the Torneträsk region in northern Sweden has been reported previously¹. This material has yielded absolutely dated

series of raw mean ring widths² and maximum latewood densities³ extending unbroken from the present back to AD 436 and AD 443, respectively. At high latitudes, both of these variables are known to correlate well with climate data (specifically, summer temperatures^{4–7}), but only qualitative inferences based on these particular long ring-width and density chronologies have been made to date^{3,8}. Here we describe the reprocessing of the raw ring-width and density data to produce new chronologies and how they have been used to derive yearly estimates of mean Fennoscandian temperatures for a statistically defined 'summer' (April–August) season, reaching back to AD 500. These are the longest annually resolved climate reconstructions from tree rings yet published.

Torneträsk tree-ring chronologies

Standardization in dendroclimatology is a method for removing variability in tree-ring series that is not related to climate. It involves removing part of the low-frequency variability from the series of raw measurements⁹. The rationale behind this is a need to account for differences in the general growth rate or vitality of individual trees and to remove trends in individual tree-ring series that arise as a consequence of non-climate-

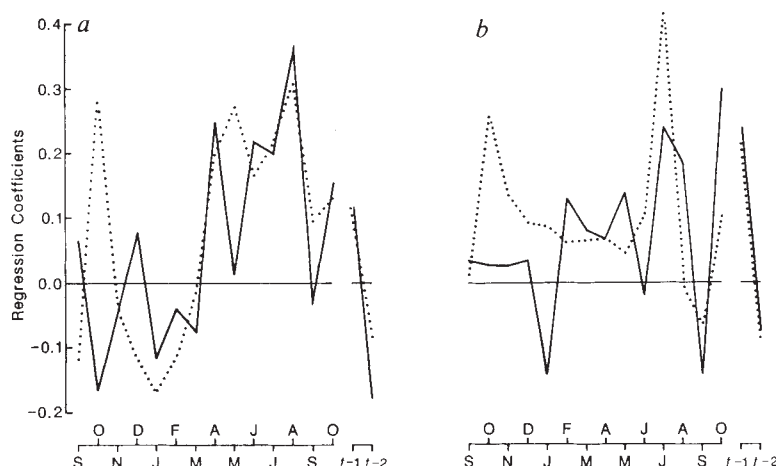


FIG. 1 Patterns of growth response (a, maximum latewood density; b, total ring width) in Torneträsk pine to intra-annual temperature forcing for the periods 1876–1925 (dotted line) and 1926–1975 (solid line), derived using multiple regression. The predictors include 14 monthly temperature series for the successive months from September of the year preceding ring formation, through to October immediately following the growing season. Two other variables, the relevant ring width or maximum latewood density for the two preceding years ($t-1$ and $t-2$) are also included to quantify the strengths of any biological preconditioning of growth in one summer by conditions in previous seasons.

related growth processes such as tree ageing or forest-stand development. Various deterministic, digital filtering and time-series approaches may be used¹⁰. Just what fraction of low-frequency variability is lost depends on the method applied. As a general rule, if efficient methods are used, the longer the constituent series, the longer is the timescale of potential climate variability that can be reconstructed from the mean standardized chronology.

The 65 ring-width and 65 maximum-density series that make up the chronologies produced here range in length from 92 to 612 years with a mean of 290 years (27 series are more than 300 years long). We have standardized the constituent data series using smoothing splines with a frequency-response cutoff set at two-thirds of the length of each series¹⁰. For example, in a 300-yr series, most of the variance above 200 years would be lost. The mean length of the splines fitted to all of the series is 194 years, but a number are long enough to preserve significant variance on timescales up to ~300 years. Although this standardization procedure effectively precludes any possibility of reconstructing climate variability on timescales much over 300 years, it is capable of preserving much of the variability on timescales approaching this.

The statistical reliability of a chronology is a function of the common growth signal (measured as the mean inter-series correlation coefficient) and the number of series (or replication depth)¹¹. The best-replicated sections of the ring-width and density chronologies (the data from the nineteenth and twentieth centuries), explain over 90% of the variance of a hypothetical infinitely replicated chronology. This figure remains above 77% back to about 550 and is only marginally worse before this. The chronologies may therefore be considered statistically reliable and homogeneous time series over virtually their whole lengths.

Temperature effects

To define an optimum season for reconstruction and to help suggest the form of linear regression equation with which to model it, we carried out temperature-response function analysis for both the ring-width and densitometric chronologies⁹. This involves regressing principal components of the monthly temperature and previous growth data on the annual tree growth indices, to derive a set of regression coefficients that indicate the direction and relative strength of the temperature forcing on tree growth. The temperature data used were area averages formed by merging gridded data from 65° and 70° N at 10°, 20° and 30° E, producing mean monthly anomalies with respect to a base period of 1951–70¹². The resulting series is considered homogeneous back to 1876.

Response functions were calculated and verified using data from 1876–1975, split into equal halves (see Fig. 1 and Table 1). Similar patterns of response are apparent over the two subperiods, although the similarity and strength of the response

is somewhat greater in the case of the densitometric data. The largest regression coefficients show that maximum density is apparently enhanced by warm conditions during all of the months from April to August. The same is generally true for ring widths, except that the regression coefficients for April, May and June are noticeably smaller than those for density and the biggest width response is in July and August. The July temperature coefficient is the largest. The importance of July temperature in the growth of high-latitude pines is well documented^{6,13,14}.

These results suggest that April–August mean ‘summer’ temperatures may be usefully reconstructed using these tree-ring data. The relatively large coefficient on the previous year’s ring width in the response function, which reflects the significant one-year-lag autocorrelation (r_1) in the ring-width series (ring width $r_1 = 0.38$, density $r_1 = 0.15$), means that a lagged ring-width variable must be included in the regression model for climate reconstruction. We therefore used a regression equation of the form

$$T_t = b_1 \text{TRW}_t + b_2 \text{TRW}_{t+1} + b_3 \text{MXD}_t + b_4 \text{MXD}_{t+1} \quad (1)$$

where T_t is the mean April–August temperature of northern Fennoscandia in year t ; TRW_t and TRW_{t+1} are the ring-width data in the years t and $t+1$ respectively and MXD_t and MXD_{t+1} are the equivalent maximum latewood densities.

Statistical reconstruction equation

Having established the potential for reconstructing the 5-month summer season (April–August) and chosen the form of regression model (equation (1)) we undertook a cross-calibration/verification exercise to test the general form of regression equation to be used for the reconstruction back to 500. The mean summer temperature series was divided into two 50-year periods 1876–1925 and 1926–1975. Equation (1) was then fitted over one period using a principal-component regression technique¹⁵ and the derived coefficients applied to the tree-growth data over the other period to give estimates of temperature which could be compared with the observed climate data (see Table 2). This process was then repeated with the periods reversed. The early- and late-calibrated regression equations are very similar with a large positive weight on current-year maximum density. The results of the verification tests show that 44–49% of the independent temperature variance is recovered in the estimated data. All of the verification tests give results that are comparable to the best tree-ring-based climate reconstructions made to date.

Having satisfied ourselves of the validity of our general regression model we re-calibrated the reconstruction equation using all 100 years of climate data so as to maximize the timescale of variability against which the final regression equation could be fitted. The recalibrated regression coefficients (Table 2) are

TABLE 1 Response function analyses on Torneträsk chronologies

			Ring width			Maximum latewood density					
			1876–1925	1926–1975	1876–1975	1876–1925	1926–1975	1876–1975			
Calibration period			1876–1925	1926–1975	1876–1975	1876–1925	1926–1975	1876–1975			
Verification period			1926–1975	1876–1925	—	1926–1975	1876–1925	—			
Calibration											
R^2			0.49	0.32	0.51	0.66	0.58	0.56			
R^2_{adj}			0.42	0.24	0.48	0.59	0.50	0.51			
Verification											
r^2			0.31	0.29	—	0.37	0.44	—			
RE			0.54	0.61	—	0.40	0.53	—			
CE			0.09	0.13	—	0.25	0.44	—			
PMt			5.3	3.6	—	3.3	4.1	—			
First difference sign											
correct			32	27	—	35	31	—			
incorrect			15	20	—	11	16	—			
Significant regression coefficients ($P=0.05$) and their signs											
Oct. ($t-1$)	+	Feb. ($t-1$)	+	Oct. ($t-1$)	+	Oct. ($t-1$)	—	Oct. ($t-1$)	—	Jan. (t)	—
Nov. ($t-1$)	+	Apr. (t)	+	Feb. (t)	+	Jan. (t)	—	Jan. (t)	—	Apr.	+
Jan. (t)	+	July	+	Mar.	—	Apr.	+	Apr.	+	May	+
Mar.	—	Oct.	+	May	+	May	+	June	+	June	+
Apr.	+			June	+	July	+	July	+	July	+
June	+			July	+	Aug.	+	Aug.	+	Aug.	+
July	+			Aug.	+	Oct.	+	Oct.	+	Density ($t-2$)	+
				Oct.	+						
Ring width ($t-1$)	+			Ring width ($t-1$)	+						

R^2 , square of the correlation coefficient calculated between actual and the estimated data; R^2_{adj} is the R^2 value adjusted to take account of the effective number of predictors; r^2 is the square of the actual/estimated correlation over the verification period; RE is the reduction of error⁸; CE of the coefficient of efficiency⁷; PMt is the t value derived using the product mean test and first difference sign is a test for comparing the signs of the first differences for the estimated and actual data⁹.

essentially the same as those for the two subperiods. The three possible reconstructions (based on the early, late and overall periods) have $\geq 95\%$ variance in common over the period 500–1980. Supporting evidence for the reliability of our temperature estimates comes from comparisons with fragmentary instrumental temperature records¹⁶. These exist for a number of sites in northern Fennoscandia. At least 20 years of data in the first half of the nineteenth century exist for the three stations, Övertorneå (66.4° N 23.8° E), Hailuoto Carlö (65.0° N 24.7° E) and Vöyri (63.2° N 22.0° E). The correlations between these early data and our reconstructed series are 0.64 (1802–1832), 0.48 (1817–1836) and 0.73 (1800–1824), respectively. These values would be expected to occur by chance one time in twenty in the case of Hailuoto Carlö and one time in a thousand for Övertorneå and Vöyri. There is also a very short early record (1830–1838) for Karesuando, which is near to the Torneträsk tree sites. The correlation between these temperature data and our reconstructions, 0.85, has a chance probability of below one in a hundred.

Summer temperature reconstructions

The complete reconstruction from 500 is plotted as temperature anomalies from the mean of 1951–70 in Fig. 2a. This long record shows that alternating periods of generally cool and generally warm conditions are typical of northern Fennoscandian summers throughout the last millennium. It is also clear that the length and amplitude of these temperature 'cycles' has varied dramatically during this time (Fig. 2b–d). There are only two periods (on a timescale of decades and above) when temperatures remained near the long-term mean: one lasting through the sixth and seventh centuries and a much shorter period in the latter half of the thirteenth century.

The smoothed line superimposed on the recent section of the reconstructed curve in Fig. 2a shows 10-year filtered values of the instrumental record for north Fennoscandia. This smooth line can be used as a modern yardstick against which to compare the magnitude and duration of summer temperature changes over the past 15 centuries. The relative warmth of the 1930s and

1940s is clear. Measured summer temperatures over these 20 years were on average 0.56 °C higher than the 1951–70 mean whereas the mean anomaly for the preceding 50 years (1880–1929) was –0.31 °C. The reconstruction shows that this recent cycle is part of a quasi 80–90 year oscillation which has continued since ~1700 (see Fig. 2d).

The mean of the reconstructed values for 1930–49 is 0.31 °C, and that for 1880–1929 is –0.20 °C. There are eight periods (based on 20-year running means) with warmth at least

TABLE 2 Calibration of reconstruction equation

Calibration Period	1876–1925	1926–1975	1876–1975
Verification Period	1926–1975	1876–1925	—
Calibration			
Explained Variance			
Overall	0.49	0.44	0.51
<10 years	0.49	0.53	0.51
>10 years	0.49	0.19	0.54
Verification			
Explained variance			
Overall	0.42	0.46	—
<10 years	0.48	0.49	—
>10 years	0.23	0.49	—
Reduction of error	0.56	0.59	—
Coefficient of efficiency	0.33	0.43	—
First-difference			
Sign test			
Correct signs	38	33	72
Incorrect signs	11	16	27
Regression weights			
(equation (1))			
b_1	–0.026	0.122	0.081
b_2	0.073	0.080	0.109
b_3	0.675	0.536	0.600
b_4	–0.014	0.113	0.052

Summary of the regression results for separate 50-year calibrations and the final reconstruction equation fitted over the full 100 years of overlap between the temperature and tree-ring data.

equivalent to 1930–49 persisting over two or more decades in the extended record, 750–780, 920–940, 960–1000, 1160–1190, 1400–1440, 1540–1570, 1750–1780 and 1840–1860. The warmest 20-year means occurred in the twelfth and the eighteenth centuries (Table 3). The second half of the twelfth century was also the warmest 50-year period (an anomaly of 0.35°C over 1152–1201). Periods of cool conditions similar to those that prevailed in the later decades of the nineteenth century include 780–830, 850–870, 1110–1160, 1330–1360, 1570–1620 and 1800–1820. Interestingly, both the warmest 20-year period and the coolest 20-year period occur in the twelfth century. Both the coolest and warmest 50-year periods occur in this century (Table 3).

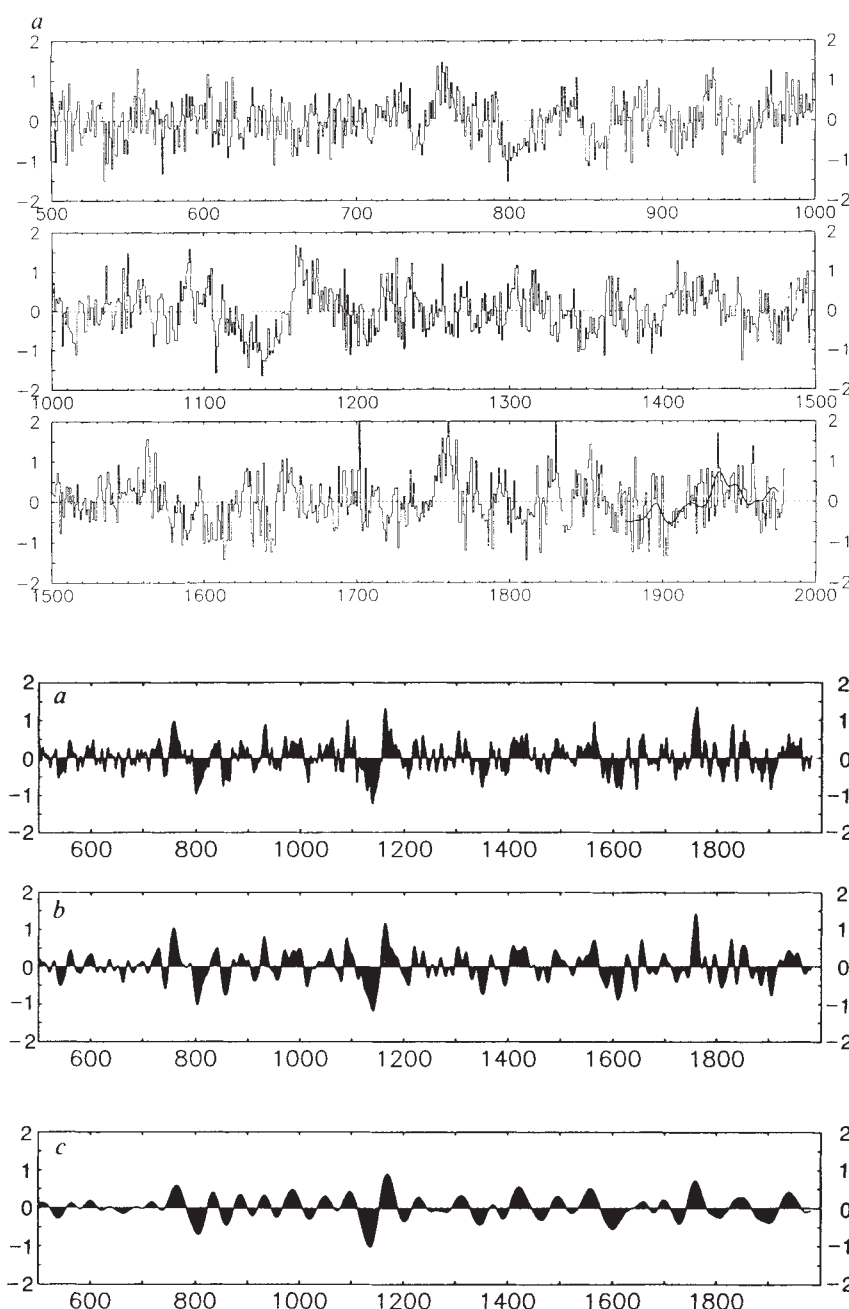
The cycle of temperature change that occurred in the twelfth century (cooling to ~ 1140 , rapid warming to ~ 1160 and subsequent cooling) is the most notable feature of the reconstruction (Fig. 2). The reconstructed anomaly of 1.59°C in 1092 was the warmest up to that date. The lowest value in the whole record, -1.65°C , occurs in 1139. Between these dates temperatures decrease rapidly and almost monotonically. The largest 50-year

cooling trend in the record, -1.78°C , occurred between 1090 and 1139. After this an even more dramatic warming took place. Within 30 years temperatures rose by $>2^{\circ}\text{C}$, with anomalies $>1.6^{\circ}\text{C}$ occurring in 1161 and 1164. The period with the largest positive 50-year trend in the reconstruction is 1127–1176 with a value of 2.43°C . From ~ 1160 temperatures again decreased linearly, a 50-year trend of -1.76°C being registered from 1161–1210 (the third largest 50-year cooling of the record). Although the largest oscillation in the record, the temperature cycle that occurred through the twelfth century is not unique. A similar cycle occurred in the second half of the eighth and the early part of the ninth centuries (Fig. 2*b–d*).

Medieval Warm Epoch and Little Ice Age

It is widely believed that two periods with strongly contrasting annual temperatures occurred during the late Holocene. The first, thought to have been a period of prolonged warmth, has been described as the Medieval Warm Epoch or the Little Climatic Optimum. It is believed to have lasted, in Europe at

FIG. 2 *a*, Reconstructed northern Fennoscandian temperature anomalies ($^{\circ}\text{C}$) (April–August mean) relative to 1951–70. Measured temperatures, smoothed with a 10-year low-pass filter, are also shown after 1876. *b–d*, The reconstructed summer temperatures after filtering with different gaussian and band-pass filters. *b*, 10-year low-pass values; *c*, variations between 25 and 40 years; *d*, variations between 50 and 150 years.



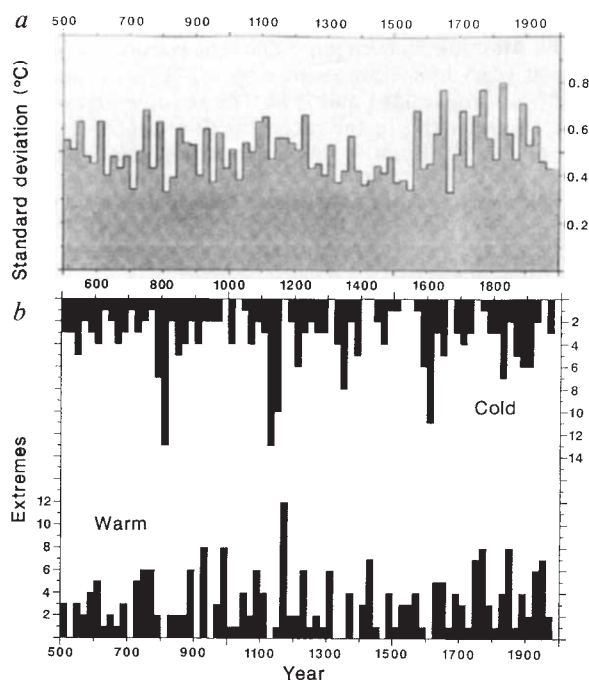


FIG. 3 *a*, Non-overlapping 20-year totals of negative and positive extremes. Extremes are defined as absolute anomalies $\geq 0.57^\circ\text{C}$ (one standard deviation of the full reconstructed series). *b*, Standard deviations calculated over the same periods.

least, from ~ 1000 to 1300 ¹⁷. Our reconstruction dispels any notion that summers in Fennoscandia were consistently warm throughout that period. Although the second half of the twelfth century was very warm the first half was very cold. For most of the eleventh and thirteenth centuries, summers were near normal (relative to the mean for 1951–70).

The other extensively cited period has been termed the Little Ice Age. During this time temperatures in many areas of both hemispheres are thought to have fallen to their lowest levels since the end of the last glaciation¹⁷. Perhaps not surprisingly, given the natural spatial variability of climate and the poor resolution of much of the data, there is confusion associated with the use of this term and there is considerable uncertainty as to the severity or synchronicity of the various cool events which have been ascribed to it¹⁸. Even in Europe the Little Ice Age has been said to have started at dates ranging between the twelfth to the sixteenth centuries and to have ended in the late seventeenth, the middle nineteenth and the early twentieth centuries¹⁹. There is, however, probably some consensus for a main European phase between 1550 and 1700 or 1800¹⁷. Our reconstruction offers strong evidence that summer temperatures in northern Fennoscandia did fall abruptly, to a level $\sim 0.5^\circ\text{C}$ below the long-term mean, at ~ 1570 . But they remained at this level only until 1650. Between 1600 and 1650, there were 13

summers with mean temperature anomalies below -1°C . This is the largest number of such cold summers in any 50-year period in the reconstruction, approached only by the 12 that occurred between 1100 and 1150. The 1550s and the 1650s, however, were warm. Conditions were near normal from 1660 to 1750 and the 20 years between 1750 and 1770 were extremely warm, the equal warmest 20 years of the whole reconstruction (Table 3).

Much of the evidence for a Medieval Warm Epoch and the Little Ice Age comes from documentary historical data¹⁷. A more direct comparison with such data can be made by using counts of extremely warm or cold summers. When this is done (Fig. 3*a*), the results lead to essentially the same conclusions. Our reconstructed summer temperatures show little evidence for a Medieval Warm Epoch in Fennoscandia and the only evidence for the Little Ice Age comes from the period ~ 1570 –1650. As regards the latter period, similar periods of cold summers occurred during the late eighth/early ninth century, the first half of the twelfth century and the second half of the fourteenth century. The first half of the twelfth century was almost certainly much more severe than the 1570–1650 period. Absence of a prolonged Medieval Warm Epoch or Little Ice Age in northern Fennoscandia is the most direct interpretation of our results. This implies either that northern Fennoscandia has experienced climate fluctuations that differed markedly from

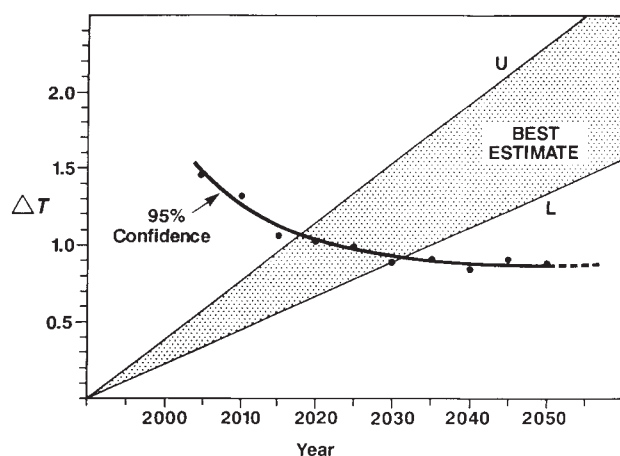


FIG. 4 The stippled area shows the 'best estimate' range of northern Fennoscandian summer temperature increase expected to result from increases in greenhouse gases (from ref. 21; some more recent estimates, unpublished, give lower values). The curved line can be considered a minimum threshold over which future summer temperature trends must pass before a greenhouse signal can be said to be distinguishable from natural climate variability at the regional scale. This detection threshold is drawn by hand through a series of empirically derived points each of which is the upper-95-percentile point of the distribution of trends of length up to that point on the horizontal axis. For example, the point plotted at 2030 (40 years after 1990) comes from the distribution of 40-year trends in the reconstruction. The illustrated threshold is a minimum because not all of the observed climate variance is reconstructed. Therefore, this graph probably provides lower-bound estimates of the detection time.

TABLE 3 Largest temperature anomalies and trends

Individual summers			
Positive		Negative	
Temperature anomaly (°C)	Year	Temperature anomaly (°C)	Year
2.07	1761	-1.65	1139
2.05	1831	-1.58	1109
1.99	1703	-1.57	961
1.71	1937	-1.53	800
1.67	1161	-1.50	536
20-year means			
Positive		Negative	
Temperature anomaly (°C)	Period	Temperature anomaly (°C)	Period
0.78	1158-1177	-0.93	1127-1146
0.78	1748-1767	-0.73	795-814
0.67	749-768	-0.65	1601-1620
0.52	1087-1106	-0.55	848-867
0.49	1551-1570	-0.53	1344-1363
50-year means			
Positive		Negative	
Temperature anomaly (°C)	Period	Temperature anomaly (°C)	Period
0.35	1152-1201	-0.58	1108-1157
0.34	1403-1452	-0.42	1576-1625
0.33	1750-1799	-0.34	780-829
0.29	719-768	-0.31	1867-1916
0.27	962-1011	-0.23	1345-1394
50-year linear trends			
Positive		Negative	
Temperature change (°C)	Period	Temperature change (°C)	Period
2.43	1127-1176	-1.78	1090-1139
1.77	1718-1767	-1.77	757-806
1.63	797-846	-1.76	1161-1210
1.17	850-899	-1.38	1560-1609
1.13	1385-1434	-1.27	1754-1803

the rest of Europe, or that the significance of these climate periods in Europe has been overstated, or both. In support of the latter possibility, we note that the proxy evidence for spatially coherent century-timescale climate fluctuations around the North Atlantic basin is relatively weak¹⁸ and the historical evidence is equally sketchy owing to problems of data reliability²⁰.

Changes in interannual variability

It is commonly believed that the Little Ice Age was marked by high interannual temperature variability. The same is often thought true of cool periods in general. We have tested this idea by calculating the standard deviations of non-overlapping 20-

year periods (Fig. 3b) and comparing them with the means for the same 20-year periods. The expected relationship would yield a negative correlation. Actually, the correlation is positive (0.23) and marginally significant at the 5% level. The three reconstructed 20-year periods with largest standard deviations (in the range 0.77–0.80 °C) all have means that are either positive or near zero. The coolest reconstructed period during the Little Ice Age (–0.65 °C in 1601–20) has a standard deviation of only 0.45 °C. Interestingly, there does seem to have been a sharp increase in the magnitude and variability of the 20-year standard deviations for the period after the second half of the sixteenth century (Fig. 3b). This is, however, strongly accentuated by the distinctly low standard deviations for the 20-year periods in the preceding 300 years.

Greenhouse-warming detectability

Our extended reconstruction allows us to evaluate possible future changes in the climate of our study region in the context of natural variability, and so to address the issue of detection of greenhouse-gas-induced climate change at the regional level. We do this by comparing expected trends in summer temperature with those that have occurred over the past 1,481 years. A recent study²¹ of possible greenhouse-gas-forced temperature change predicts that between 1990 and 2030 the global mean temperature will increase by 0.4–2.3 °C, with a 'best estimate' in the range 1.0–1.7 °C. Another study, integrating the regional patterns of climate change produced in a number of doubled CO₂ experiments using equilibrium general circulation models, suggests that the summer temperature change in northern Fennoscandia is likely to be somewhat less than this, between 0.75 and 1.0 times the annual mean global change²². Therefore, a rough estimate of the expected summer temperature trend in northern Fennoscandia resulting from the greenhouse effect is somewhere between ~0.9 and 1.5 °C over the next 40 years.

We have used our reconstruction to generate the observed frequency distributions of trends over periods of different length up to 60 years, using only those trends that originate from the currently prevailing level, that is, within a range of temperatures 0.1 °C on either side of the long-term mean. This avoids distortions that may arise from warming and cooling trends that represent 'recovery' from very anomalous conditions. Plotting the upper-95-percentile point from each of the observed distributions of 15-, 20-, 25-, and so on, year trends, along with the range of predicted local greenhouse warming allows us to estimate when the predicted changes will rise above the 'noise' of natural variability (Fig. 4).

Even if the climate sensitivity is at the top of the generally accepted range (U in Fig. 4), it will still not be possible to detect a regional greenhouse signal (with 95% confidence) until around 2020. If climate sensitivity is at the lower end of the range (L), as is indicated by the empirical evidence of recorded global temperatures over recent decades²³, then we will be unable to identify the signal in Fennoscandian temperatures with high confidence until some time after the year 2030. □

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Transient folding intermediates characterized by protein engineering

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Kinetic experiments on engineered mutants of barnase detect an intermediate on the folding pathway and allow the mapping of the tertiary interactions of the side chains and their energetics. Many of the interactions present in the final folded state tend to be either fully formed or not formed at all in the intermediate or subsequent transition state for folding, but the hydrophobic core becomes progressively consolidated. These methods in combination with NMR provide extensive structural characterization of the folding intermediate and the sequence of events in the folding pathway.

THE pathway of protein folding is dominated by noncovalent interactions that determine structure and stability. There is growing evidence that the refolding of proteins *in vitro* proceeds through intermediates that can accumulate transiently¹⁻⁵. Unlike intermediates in covalent chemistry, which can frequently be isolated and characterized because of the relatively stable chemical bonds, the intermediates in protein folding, aside from -S-S- linkages, differ just in noncovalent bonding. The ephemeral nature of such bonds causes severe problems in the characterization of folding intermediates and requires novel methods for their investigation⁶⁻⁹. A recent high-resolution approach uses NMR, which can detect backbone NH groups that undergo hydrogen exchange slowly with solvent because they are hydrogen bonded within α -helices, β -sheets or other structural elements. Rapid quenching experiments on hydrogen exchange during refolding can detect secondary structure that is formed faster than the final folded state. For example, regions of β -sheet and α -helix respectively, are formed rapidly in the refolding of RNase A (ref. 6) and cytochrome *c* (ref. 7). Earlier work also shows intermediate states. Spectroscopy and light and X-ray scattering measurements on α -lactalbumin have shown the existence of a state, termed the molten globule, which is stable under mildly denaturing conditions (acid pH, moderate concentrations of denaturants or high temperatures¹⁻⁵). This state has significant secondary structure and is compact, but has a fluctuating hydrophobic core. It is difficult to study by NMR because of broad linewidths and extensive overlapping of peaks but methods have been developed to confirm some of its characteristics⁸. An intermediate state similar to or identical with the molten globule, has been suggested as a general occurrence on folding pathways^{10,11}.

We have applied protein engineering to analyse the unfolding of barnase, a small RNase from *Bacillus amyloliquefaciens*, and have shown how this approach can characterize the noncovalent structural changes in the transition state, which is inaccessible to the usual spectroscopic methods¹². Mutations were made that remove individual interactions stabilizing different regions of the folded structure. Each mutation acts as a specific probe for

structural changes during unfolding. We show here how kinetics can detect a transiently formed intermediate in the refolding pathway and present the full theory of how the protein engineering procedure can characterize structural properties of the intermediate that cannot be detected by NMR-hydrogen-exchange, such as events in the hydrophobic core or in hydrogen bonds that are in fast exchange, in addition to giving indirect evidence on secondary structure. The procedure also gives the energetics of noncovalent interactions within these conformations. The kinetic studies are complemented and reinforced by a separate study in which NMR-H-exchange experiments identify further secondary structure in the intermediate¹³.

Kinetics detects folding intermediate

From experiment, the logarithm of the equilibrium constant for the unfolding of small proteins in urea (K_u) is proportional to the urea concentration¹⁴:

$$\log K_u = \log K_u^{\text{H}_2\text{O}} + m_c[\text{urea}] \quad (1)$$

where $K_u^{\text{H}_2\text{O}}$ is the equilibrium constant for unfolding in water

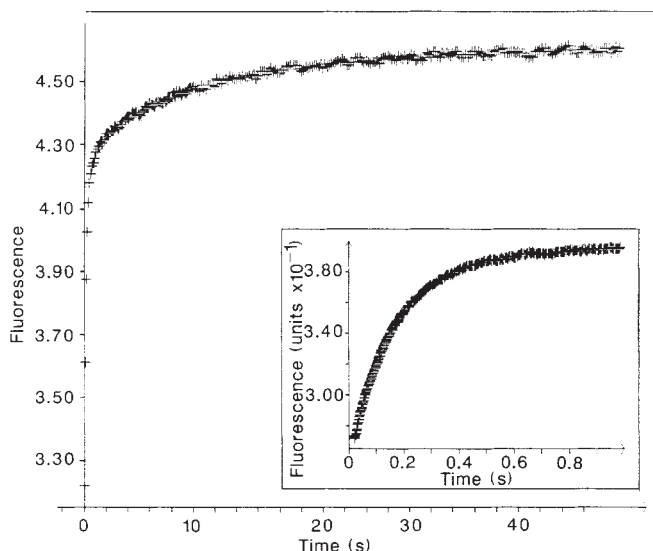


FIG. 1 Refolding of wild-type barnase monitored by increase in tryptophan fluorescence, excitation at 290 nm and emission at 315 nm. Refolding was initiated by mixing protein denatured in 7 M urea, 50 mM MES buffer, pH 6.3 with 10 volumes of 50 mM MES, pH 6.3 at 25 °C to give 0.636 M urea, using a Perkin-Elmer spectrofluorimeter MPF 44B fitted with a stopped-flow mixer¹². The time scale for the slower phase is 0 to 50 s. Inset, fast phase from 30–950 ms. The fast phase has a rate constant of 6.5 s^{-1} and amplitude 0.131 units whereas the slow phase increases at 0.094 s^{-1} with amplitude 0.032 units. The rate constant for the slow phase varies only within a range of $\pm 20\%$ for different mutants under the same conditions. The thickness of the trace does not represent the noise in the signal but is an artefact of the symbols used by the plotter. The signal-to-noise ratio is better than 50:1. The amplitude of the slow phase is that expected if $\sim 5\%$ of each peptidyl prolyl linkage is *cis*. A value of 0.044 to 0.053 has been recently reported for the equilibrium constant for *cis/trans* Lys 116–Pro 117 in unfolded staphylococcal nuclease^{24,25}.

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and m_e is a constant that is proportional to the increase in degree of exposure to solvent of the protein on denaturation. The rate constant for unfolding, k_u , is generally found to increase with increasing urea concentration according to equation (2) where $k_u^{\text{H}_2\text{O}}$ is the rate constant in H_2O (refs. 5,12–15).

$$\log k_u = \log k_u^{\text{H}_2\text{O}} + m_{ku}[\text{urea}] \quad (2)$$

Equations (1) and (2) imply that the rate constant for folding, k_F , of a protein that involves the reversible transition between just two states, E_F and E_U , (equation (3))



must follow the rate law

$$\log k_F = \log k_F^{\text{H}_2\text{O}} - (m_e - m_{ku})[\text{urea}] \quad (4)$$

(As $\log k_F^{\text{H}_2\text{O}} = \log(k_u^{\text{H}_2\text{O}}/K_u^{\text{H}_2\text{O}})$, the value of $\log k_F$ may be calculated simply from the unfolding rate constants and equilibrium data.)

The kinetics of protein folding is often complicated by the slow and frequently rate-determining isomerization of proline residues¹⁶. Barnase has three proline residues, which are all *trans* in the native structure. Earlier work suggests that some 5–10% of each of the prolines in the denatured protein should exist in the *cis* conformation and that the conformations should interconvert with a half-life of tens of seconds and an activation energy of about 15 kcal mol⁻¹ (ref. 17). We find that when barnase that has been incubated in 6–8 M urea is diluted with 10 volumes of water there is a biphasic regain of folded structure with the two kinetic phases well resolved (Fig. 1). The first phase, 83% of the amplitude measured by stopped-flow fluorescence spectroscopy¹², occurs with a half-life about 50 ms at low urea concentration that increases with increasing urea. The slow phase, 17% of the amplitude, has a half-life of 10–20 s, changes only slowly with urea concentration and has an activation energy of about 13 kcal mol⁻¹. These are just the characteristics expected for the conversion of ~5–10% each of three proline residues in the *cis* conformation in the unfolded enzyme, to *trans* in the folded. (This interpretation has subsequently been confirmed directly by showing that the slow phase is efficiently catalysed

by peptidyl prolyl isomerase, L. Meiering, unpublished results). The fast and main phase is the folding of the fraction of unfolded protein that has all its prolines in the *trans* conformation in solution. Measurements of regain of activity on renaturation demonstrate that the fast phase corresponds to the formation of active enzyme. The following discussion refers just to the fast phase.

Figure 2 shows that whereas the simple rate law of equation (1) describes the unfolding of wild-type barnase, $\log k_F$ does not follow equation (4). The value of $\log k_F$ at low urea concentration deviates by many orders of magnitude from that predicted by the kinetics of a two-state equation. This is classic evidence for the existence of a metastable intermediate on the refolding pathway: the intermediate accumulates on a fast time scale and the observed rate constant is that for the reaction of the intermediate and not for its formation. The deviation from ideality is not caused by the equilibrium and rate equations (1) and (2) breaking down at lower concentrations of urea. The evidence for this is: $\log k_u$ can be measured with suitable mutants under more acidic conditions down to 0.5 M urea and is linear with urea concentration over the relevant concentration range (Fig. 2); $K_u^{\text{H}_2\text{O}}$ measured from the linear extrapolation of

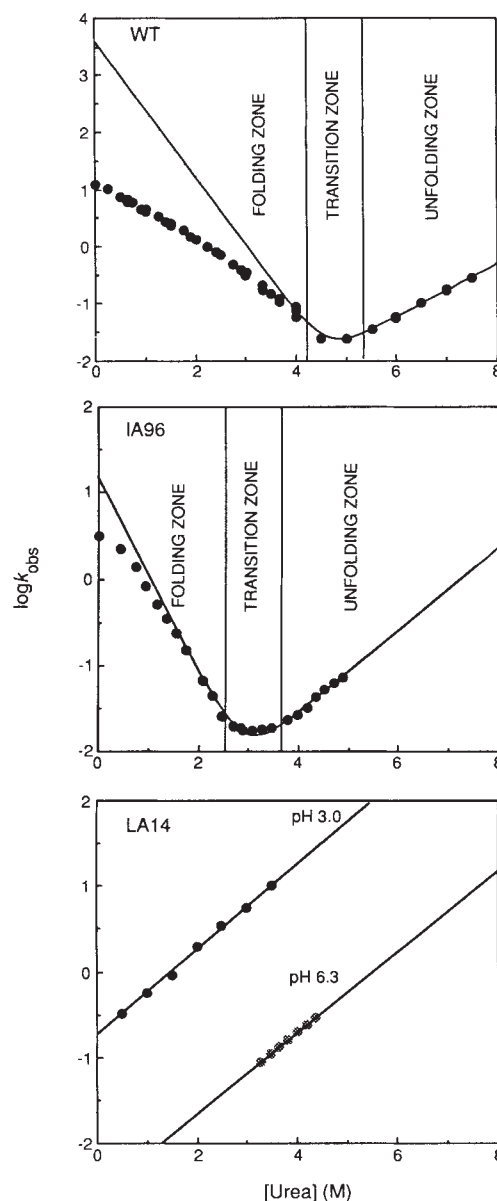


FIG. 2 Urea dependence of rate constants for denaturation and renaturation of wild-type and mutant enzymes. Rate constants are measured in units of s^{-1} at 25 °C, pH 6.3 and 50 mM MES buffer. Upper panel, $\log k_{\text{obs}}$ for folding of wild-type enzyme. The experiments at low concentrations of urea consist of solutions of enzyme in high concentrations of urea diluted 11-fold into refolding buffer in the stopped-flow fluorimeter¹². In this region, k_{obs} is the rate constant for refolding. (The point at 0 M urea was obtained from the renaturation of acid-denatured enzyme, after a pH jump from low pH.) At high urea concentration, folded enzyme in aqueous buffer was mixed with 10 volumes of urea solution, to observe the kinetics of unfolding. In the transition region, both folded and unfolded protein exist at measurable concentrations. The rate constants in this region may be measured by mixing either unfolded or folded protein with urea and monitoring the progress to equilibrium ($k_{\text{obs}} = k_u + k_F$ for a simple two-state transition when both forward and reverse rate constants are significant)²⁶. The solid curve is that calculated for a simple two-state system (equation (3)) and $\log k_{\text{obs}} = \log(k_u + k_F)$ (ref. 26). The rate constant k_u was calculated from the experimental values for the unfolding kinetics, which conform to equation (2) with $k_u^{\text{H}_2\text{O}} = 1.1 \times 10^{-4} \text{ s}^{-1}$ and $m_{ku} = 0.463 \text{ M}^{-1}$. k_F was calculated by inserting the value of k_u into equation (1) using the values of $K_u^{\text{H}_2\text{O}}$ and m_e from equilibrium unfolding measurements (The two-state approximation holds in the transition region). Middle panel, $\log k_{\text{obs}}$ for the mutant IA96 (Ile → Ala). The two-state approximation holds nicely down to less than 2 M urea and well below the transition region. The solid curve was calculated as above using $k_u^{\text{H}_2\text{O}} = 3.2 \times 10^{-4} \text{ s}^{-1}$ and $m_{ku} = 0.479 \text{ M}^{-1}$. Bottom panel, $\log k_u$ measured at low concentrations of urea for the destabilized mutant LA14 (Leu → Ala). The stability of barnase decreases at low pH (ref. 27). At pH 3.0 LA14 is >95% unfolded at 0.5 M urea. LA14 was dialysed against distilled water at pH 7 and mixed in the stopped-flow fluorimeter, as above, with 10 volumes of 110 mM sodium formate, pH 3.0, containing various concentrations of urea. The data for pH 6.3 are given for comparison.

FIG. 3 Thermodynamic analysis. Top, Cycles relating properties of mutant and wild-type enzymes. The energy difference between E_F and E_U , ΔG_{F-U} ($=G_{E_F} - G_{E_U}$), is measured from equilibrium unfolding. The energy difference between E_F and E_I , ΔG_{F-I} ($=G_{E_F} - G_{E_I}$), is calculated from the ratio k_u/k_{-u} . ΔG_{I-U} ($=G_{E_I} - G_{E_U}$) is calculated from $\Delta G_{F-U} - \Delta G_{F-I}$. The free-energy of the transition state, $\Delta G_{\ddagger-F}$ ($=G_{E_{\ddagger}} - G_{E_F}$), can be calculated from transition state theory²⁶ and the value of k_u , the rate constant of unfolding¹². $\Delta G_{\ddagger-U}$ ($=G_{E_{\ddagger}} - G_{E_U}$) is calculated by adding $\Delta G_{\ddagger-F}$ and ΔG_{F-U} . There may be uncertainties in calculating the absolute values of $\Delta G_{\ddagger-F}$ by transition state theory but the errors cancel out when measuring $\Delta\Delta G_{\ddagger}$, the difference in $\Delta G_{\ddagger-F}$ between wild-type and mutant enzymes, as only ratios of rate constants are used. For wild-type enzyme, the following values are calculated in H₂O at 25 °C and pH 6.3: $\Delta G_{I-U} = 3.2 \text{ kcal mol}^{-1}$; $\Delta G_{\ddagger-U} = 9.6 \text{ kcal mol}^{-1}$; and $\Delta G_{F-U} = -10.2 \text{ kcal mol}^{-1}$. In the vertical virtual equilibria, ΔG_U is the difference in noncovalent energy between unfolded wild-type and mutant enzymes, $\Delta G_I = G_{E_I} - G_{E'_I}$ is the difference between the folding intermediates, $\Delta G_{\ddagger}$ is the difference between the transition states and ΔG_F between the folded states. Bottom, Calculation of difference energies. The difference energies are defined as: $\Delta\Delta G_F = \Delta G_{F-U} - \Delta G'_{F-U}$; $\Delta\Delta G_{\ddagger} = \Delta G_{\ddagger-U} - \Delta G'_{\ddagger-U}$; $\Delta\Delta G_I = \Delta G_{I-U} - \Delta G'_{I-U}$ where the prime denotes mutant. In practice, all energies are calculated as difference energies and relative to the folded state ($\Delta\Delta G_{F-U}$, $\Delta\Delta G_{F-I}$, $\Delta\Delta G_{F-\ddagger}$) and then converted to the difference energies relative to the unfolded state by subtracting them from $\Delta\Delta G_{U-F}$. The difference energies are the experimentally determined values. These may be related to the true differences in noncovalent energy between wild-type and mutant proteins by extending theory developed previously^{12,21} (see text). The difference energies may be measured as above with high precision. $\Delta\Delta G_F$ measured from equilibrium denaturation is reproducible to $\pm 0.03 \text{ kcal mol}^{-1}$. The value of $k_u^{\text{H}_2\text{O}}$ is measured to better than $\pm 5\%$ (standard error). The precision of values of $k_u^{\text{H}_2\text{O}}$ vary from a standard error of $\pm 14\%$ for wild-type from the extrapolation to 0 M urea of experiments conducted at concentrations above 5.5 M, to $\pm 2\%$ for the shorter extrapolation of unfolding data for a destabilized mutant such as LA14 (see Fig. 2). The consequent errors in $\Delta\Delta G_{\ddagger}$ and $\Delta\Delta G_I$ are estimated from the method of calculation to be less than $\pm 0.14 \text{ kcal mol}^{-1}$. The differences between $\Delta\Delta G_{\ddagger}$, $\Delta\Delta G_I$ and

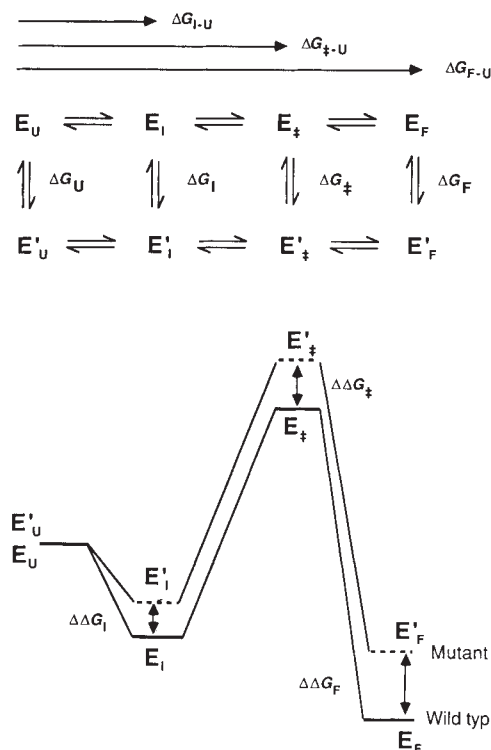
measurements of $\log K_u$ at high urea concentration agrees with that measured by temperature- or guanidinium chloride-induced denaturation¹⁸; the folding kinetics of some less stable mutants follow the two-state equation down to much lower concentrations of urea because the intermediate is destabilized and accumulates to a much smaller extent (for example, mutant IA96 in Fig. 2).

It has been suggested that the deviation in the rate constants for refolding is not a consequence of an intermediate accumulating but is caused by the *cis* $\rightarrow$ *trans* isomerization of one of the proline residues becoming rate determining at low urea. This is unlikely for the following reasons. First, the amplitude of the fast phase (83%) is so high that it would require the *cis* conformation to be the major form in the unfolded enzyme. Second, the rate constant of 13 s^{-1} is far too high for a *cis*–*trans* isomerization of a proline in a denatured protein. Third, the high amplitude accounts for most of the reaction and so there would still be a discrepancy between the equilibrium constant for unfolding calculated from the ratio of rate constants and that from calculated equilibrium studies.

The NMR study¹³ shows directly that on the folding pathway there is an intermediate with considerable formation of secondary structure. The refolding of wild-type enzyme is a multi-phasic reaction of which we measure only the slowest steps in the stopped-flow experiments. The faster steps are lost in the dead time of the stopped-flow spectrophotometer, which is 30 ms because of problems in mixing urea with water. The slowest step is the isomerization of prolines in the denatured state. This step has the same rate constant for all the mutants studied. The following analysis of the kinetic consequences of the folding intermediate on the refolding pathway applies to the phase that is observed in the stopped-flow time range before the *cis*–*trans* isomerization.

Free energy profile for folding

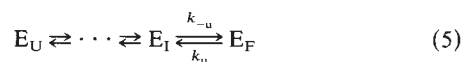
The role of binding energy of many groups at the active site of the tyrosyl-transfer RNA synthetase was elucidated by compar-



$\Delta\Delta G_F$ are even more precisely known because $\Delta\Delta G_{\ddagger}$ and $\Delta\Delta G_I$ are measured relative to $\Delta\Delta G_F$. The error in $\Delta\Delta G_{\ddagger} - \Delta\Delta G_F$ and in $\Delta\Delta G_I - \Delta\Delta G_F$ is less than $\pm 0.11 \text{ kcal mol}^{-1}$.

ing the free-energy profiles of wild-type and mutant enzymes throughout the reaction¹⁹. What is crucial is not so much the free-energy profile itself but the differences in energy levels between mutant and wild-type enzymes. The 'difference energy' diagrams readily give a qualitative picture of the importance of each side chain throughout the reaction^{19,20} and the results can be interpreted quantitatively²¹. The same approach may be applied to study the energetics of interactions of side chains during protein folding.

First, the free-energy profile of the folding pathway of the wild-type protein must be (partly) constructed. The kinetics of folding from 30 ms fit, with high precision, a single exponential trace with a slow tail for proline isomerization¹³. There may be many intermediates on the reaction pathway. Some of these occur before the formation of the one observed, E_I , but are lost in the dead time of the apparatus because they are formed and react too fast. Others will occur between E_I and E_F but are after the rate-determining step for folding (see discussion in ref. 12). The observed rate constant, k_{-u} , is simply that for E_I transforming to E_F (equation (5)). The observed rate constant for unfolding, k_u (equation (2)), is the rate constant for the formation of E_I from E_F , which is the rate-determining step for unfolding.



(If ' E_I ' is a mixture of intermediates, then k_{-u} is a weighted mixture of rate constants, and reports back on the weighted average of the properties of the mixture.)

The schemes for analysing the kinetics and equilibria are given in Fig. 3. The analysis is in terms of difference energy diagrams or apparent energy differences. The free-energy profile for folding of wild-type enzyme is calculated from the measured free energy of unfolding and the rate constants $k_u^{\text{H}_2\text{O}}$ and $k_{-u}^{\text{H}_2\text{O}}$, the values in the absence of denaturant, to give the energies of the folded state, transition state for unfolding and the intermediate relative to the unfolded state. The unfolded state is assigned

a standard energy of 0. The same is done for a mutant. An accurate procedure for calculating the differences in the energy levels is given in the legend to Fig. 3.

Difference energy diagrams

In Fig. 4 are plotted the difference energy diagrams for a variety of mutants, each chosen as before¹², to remove a small interaction that stabilizes a specific part of the structure. The crucial step in the interpretation is to relate the experimentally measured difference energies ($\Delta\Delta G_I$, $\Delta\Delta G_{\ddagger}$ and $\Delta\Delta G_F$ in Fig. 3) to the true differences in noncovalent energy between each of the states. The true differences are defined as:

$$\Delta G_U = G_{E_U} - G_{E'_U}, \quad \Delta G_I = G_{E_I} - G_{E'_I}, \\ \Delta G_{\ddagger} = G_{E_{\ddagger}} - G_{E'_{\ddagger}} \quad \text{and} \quad \Delta G_F = G_{E_F} - G_{E'_F}$$

(where G_{E_U} is the free energy of unfolded wild-type enzyme, $G_{E'_U}$, that of unfolded mutant, and so on). Applying the first law of thermodynamics to the cycle in Fig. 3 gives:

$$\Delta\Delta G_F = \Delta G_F - \Delta G_U \quad (6)$$

$$\Delta\Delta G_{\ddagger} = \Delta G_{\ddagger} - \Delta G_U \quad (7)$$

$$\Delta\Delta G_I = \Delta G_I - \Delta G_U \quad (8)$$

The apparent energies (' $\Delta\Delta G$ ') differ from the true by a constant term for each mutant, ΔG_U , the difference in free energy between unfolded mutant and wild-type enzymes. An important corollary of equations (6)–(8) is that the change in the experimentally determined apparent energies on going from one state to another in the folding profile is equal to the difference in true energies. For example, from equations (6) and (7):

$$\Delta\Delta G_F - \Delta\Delta G_{\ddagger} = \Delta G_F - \Delta G_{\ddagger} \quad (9)$$

and from equations (7) and (8):

$$\Delta\Delta G_{\ddagger} - \Delta\Delta G_I = \Delta G_{\ddagger} - \Delta G_I \quad (10)$$

Changes in apparent energies ($\delta\Delta\Delta G_{app}$) are always equal to changes in true stabilization energies ($\delta\Delta G_{true}$) along a profile, that is, $\delta\Delta\Delta G_{app} = \delta\Delta G_{true}$. Equations (6)–(10) hold for all mutations in all circumstances. But they apply to the global energies of the different states of wild-type and mutant enzymes. The crucial question in the interpretation of these equations is, therefore, whether the changes in energy relate just to the local changes in bond energies around the site of mutation. It is possible that $\delta\Delta\Delta G_{app}$ has a significant contribution from longer range global changes that vary from state to state between wild type and mutant. This can be analysed by dividing the change in noncovalent energy on mutation of a side chain into two terms: one, ΔG_{local} , arising from changes where the mutated side chain was previously in contact with other residues; and a second, ΔG_{reorg} , the reorganization energy²¹, arising from changes in the energy of the protein elsewhere, in both conformational energy and solvation. Thus, $\delta\Delta\Delta G_{app} = \delta\Delta G_{local} + \delta\Delta G_{reorg}$. If $\delta\Delta G_{reorg} = 0$, then, $\delta\Delta\Delta G_{app}$ reports back on just the local events during folding. If, $\delta\Delta G_{reorg} \neq 0$, then $\delta\Delta\Delta G_{app}$ is uninterpretable in a simple manner.

The two major assumptions in this study are: the mutations are such that ΔG_{reorg} and $\delta\Delta G_{reorg}$ are small and do not obscure the results; and the mutations do not perturb the pathway of folding. This achieved by using two categories of mutation. The first is deletion where a portion of a side chain is removed without introducing a new function (such as Ile $\rightarrow$ Val, Tyr $\rightarrow$ Phe). If there are no changes in protein structure on mutation other than removal of part of the side chain, the mutation is said to be nondisruptive²⁰ and $\delta\Delta G_{reorg}$ is zero. Under these conditions, $\delta\Delta\Delta G_{app} = \delta\Delta G_{local}$. Further, where there is an empty cavity in the mutant enzyme at the site of deletion, $\delta\Delta G_{local}$ is a direct measure of the interaction energy of the side chain in the wild-type enzyme with the contiguous residues. Mutations that cause an increase in the size of a buried side

chain or add new contacts are avoided as they are likely to lead to disruptions of structure and the high probability that $\delta\Delta G_{reorg} \neq 0$.

The second category is mutation of surface residues. Here, solvent can move to accommodate changes. Further, for changes at the surface where there is free access of water to the mutated sites, $\Delta\Delta G_{app}$ approximates to the true binding energy of the group that is deleted²¹. We prefer to make mutations that give relatively small changes in stabilization energy as not only are they likely to cause insignificant values of ΔG_{reorg} and $\delta\Delta G_{reorg}$, they are also the least likely to perturb the pathway of folding.

We find for many of the mutations analysed in this study that there is a greatly simplifying feature: the change in stability of the intermediate or transition state on mutation is either close to zero or close to 100% of that of the change in stability of the folded enzyme. These are the changes expected if the interactions are, respectively, either not formed at all or fully formed in the intermediate or transition state.

The difference energies are calculated with high precision. For each mutant relative to wild type, the error in $\Delta\Delta G_F$ is less than ± 0.03 kcal mol⁻¹, and in $\Delta\Delta G_{\ddagger}$ and $\Delta\Delta G_I$ less than ± 0.14 kcal mol⁻¹ (see legend to Fig. 3). Relative to $\Delta\Delta G_F$, $\Delta\Delta G_{\ddagger}$ and $\Delta\Delta G_I$ are known to an accuracy of better than ± 0.11 kcal mol⁻¹ as the error in $\Delta\Delta G_F$ affects the three energy terms equally. Problems in interpreting difference energies thus do not arise from uncertainties in the accuracy of the measurements.

Analysis of mutants

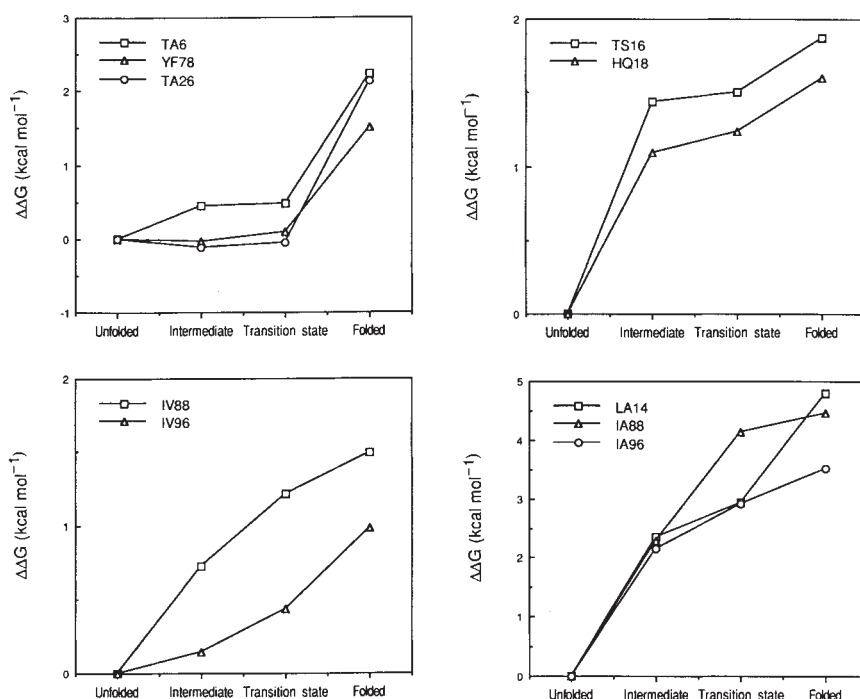
YF78, TA6, TA26—probing a loop and the N termini of helices. Tyr 78 stabilizes a major loop by its -OH group forming hydrogen bonds with the NH and C=O of Gly 81 in the folded state (Fig. 5). Mutation of Tyr $\rightarrow$ Phe (YF78) is a deletion that should not cause significant structural changes. It was shown previously¹² that in this mutant all the stabilization energy from the hydrogen bonds is lost in the transition state for unfolding. All the energy is lost in the intermediate as well: $\delta\Delta\Delta G_{app}$ is almost zero between E_I and $E_{\ddagger}$. The -OH of Thr 26 acts as the N-cap of the second helix, forming hydrogen bonds to the NH groups of residues 27–29 (ref. 22). Mutation of the N-cap to Ala (TA26) is a good mutation to analyse as it is simply an alteration of a surface residue that is replaced by solvent water. Again, it was shown previously by mutating TA26 that all the energy of the N-cap is lost in the transition state for unfolding and the same is seen to happen in the intermediate. Thr 6 forms the N-cap of the first helix. In the mutant TA6 (Thr $\rightarrow$ Ala) 80% of the difference energy is lost in the transition state for unfolding, and the same amount of energy is lost in the intermediate as in the transition state.

HQ18, TS16—probes of the major α -helix. The charge on the protonated form of His 18 makes a coulombic interaction with the dipole of the α -helix from residues 6–18, and an imidazole NH makes a hydrogen bond with the backbone CO of Gln 15. The interaction energy is thus a probe of the integrity of the C terminus of the helix. In the mutant HQ18 (His $\rightarrow$ Gln), most of this interaction energy is maintained in the transition state¹². It is maintained equally in the intermediate. A second residue that probes the C terminus is Thr 16. The γ -methyl of Thr 16 makes a very strong hydrophobic interaction with the aromatic ring of Tyr 17, the next residue in the helix in the folded structure. In the mutant TS16 (Thr $\rightarrow$ Ser) this is maintained in the transition state for unfolding¹², and is seen now to be equally maintained in the intermediate, adding further evidence for early helix formation. These mutations are most suitable as they alter just surface side chains that are exposed. Under these circumstances, values of $\Delta\Delta G_{app}$ are close to the true binding energies²¹.

LA14, IA88, IA96, IV88, IV96—probes of the hydrophobic core. The principal α -helix (residues 6–18) packs onto the antiparallel β -sheet by the interdigitation of hydrophobic side chains, of which Leu 14, Ile 88 and Ile 96 are representative, to form a hydrophobic core. Mutation of these differs from the previous

FIG. 4 Difference energy diagrams. The difference energies, $\Delta\Delta G_{app}$, calculated according to Fig. 3 are plotted for the mutants. The following rate constants and equilibrium data have been used. Wild type: $k_{H_2O}^{H_2O} = 1.1 \times 10^{-4} \text{ s}^{-1}$, $k_{H_2O}^{H_2O} = 13 \text{ s}^{-1}$, TA6: $k_{H_2O}^{H_2O} = 2.2 \times 10^{-3} \text{ s}^{-1}$, $k_{H_2O}^{H_2O} = 15 \text{ s}^{-1}$, TA26: $k_{H_2O}^{H_2O} = 4.6 \times 10^{-3} \text{ s}^{-1}$, $k_{H_2O}^{H_2O} = 14 \text{ s}^{-1}$, YF78: $k_{H_2O}^{H_2O} = 1.2 \times 10^{-3} \text{ s}^{-1}$, $k_{H_2O}^{H_2O} = 13 \text{ s}^{-1}$, TS16: $k_{H_2O}^{H_2O} = 2.1 \times 10^{-4} \text{ s}^{-1}$, $k_{H_2O}^{H_2O} = 14 \text{ s}^{-1}$, HQ18: $k_{H_2O}^{H_2O} = 2.1 \times 10^{-4} \text{ s}^{-1}$, $k_{H_2O}^{H_2O} = 12 \text{ s}^{-1}$, LA14: $k_{H_2O}^{H_2O} = 2.7 \times 10^{-3} \text{ s}^{-1}$, $k_{H_2O}^{H_2O} = 5.6 \text{ s}^{-1}$, IV88: $k_{H_2O}^{H_2O} = 1.8 \times 10^{-4} \text{ s}^{-1}$, $k_{H_2O}^{H_2O} = 6.5 \text{ s}^{-1}$, IA88: $k_{H_2O}^{H_2O} = 2.0 \times 10^{-4} \text{ s}^{-1}$, $k_{H_2O}^{H_2O} = 0.7 \text{ s}^{-1}$, IV96: $k_{H_2O}^{H_2O} = 2.9 \times 10^{-4} \text{ s}^{-1}$, $k_{H_2O}^{H_2O} = 9.2 \text{ s}^{-1}$, IA96: $k_{H_2O}^{H_2O} = 3.2 \times 10^{-4} \text{ s}^{-1}$, $k_{H_2O}^{H_2O} = 4.1 \text{ s}^{-1}$.

Identical values for $k_{H_2O}^{H_2O}$ are obtained from either extrapolation of the rate constants for refolding at low urea concentrations to 0 M or directly from renaturation by a pH jump from pH 1.5 to 6.3 in the absence of urea. $k_{H_2O}^{H_2O}$ is obtained by linear extrapolation (see data in ref. 12). A referee has noted that the values of $\Delta\Delta G_F$ for just the interactions measured here account for most of the observed free-energy of folding of the protein ($\Delta G_{F-U} = -10.2 \text{ kcal mol}^{-1}$). This is not anomalous because, as defined in Fig. 3, $\Delta G_{F-U} = G_F - G_U$. G_F and G_U are both much larger than ΔG_{F-U} , possibly being in the region of thousands of kcal mol^{-1} . Thus, the values of $\Delta\Delta G_F$ are $<1\%$ of G_F . Whether or not a protein folds depends on the small difference between G_F and G_U . Just a few mutations can tip



the balance so that the unfolded form is favoured at equilibrium.

examples as there are obvious progressive changes in the difference energies as the protein folds (Fig. 4). Mutation of these apolar side chains should lead to small values of ΔG_U (ref. 12) and so changes in difference energies most probably do reflect the true energy changes. Mutations of isoleucines to valines (IV88, IV96) are better probes than mutation to alanine (IA88, IA96) as the smaller the mutational change the less the perturbation of structure. Ile $\rightarrow$ Val is almost an ideal mutation whereas Ile $\rightarrow$ Ala leaves a larger cavity. Surrounding residues could relax into the cavity with some probability of a significant reorganization energy term, or there could be ingress of solvent. For IV96 and IV88, there is a clear progression of $\Delta\Delta G_{app}$ as the reaction proceeds, and the same is true for the larger mutations. There are individual variations in the energetics but, in

all cases, the stabilization of the intermediate is about midway between that of the unfolded and folded states whereas the transition state has up to 80–90% of the final stabilization. The individual variation might reflect the differences in binding of the different methylene groups in each side chain or could be a consequence of ΔG_{reorg} terms. Nevertheless, it is clear that the hydrophobic core becomes consolidated as folding proceeds.

Reliability of structural conclusions

The deductions about changes in structure from changes in energetics are, of course, indirect. Aside from the assumptions that $\delta\Delta G_{reorg}$ is small and the mutations do not perturb the pathway of folding, what other problems could there be to obscure the interpretations? The results can be divided into two extreme classes to illustrate these: those where interactions are maintained on changing state and those where interactions are lost. Maintenance of an interaction energy does not prove that the particular interaction itself is maintained; it could be that a different interaction of equivalent energy is formed in the new state. Total loss of an interaction energy in an intermediate, however, is conclusive evidence for the loss of an energetically significant interaction as there is not the ambiguity of an alternative interaction. The evidence for loss of the N-caps during the unfolding of barnase is, thus, unambiguous. Evidence for maintenance of the interactions at the C terminal of the major helix is very good. Two separate mutations, based on different properties of the helix, give consistent results, as do NMR experiments on hydrogen exchange¹³. Results on the hydrophobic core are less conclusive; the changes in interaction are intermediate between the two classes. The core is unambiguously weakened in the transition state and intermediate, but it could be changed even more than indicated by the mutational data as there could be alternative hydrophobic interactions taking place.

Intermediate and folding events

Protein engineering on Thr 16 and His 18 shows that the C-terminal portion of the α -helix extending from residues 6–18 is formed in the intermediate. N-capping of the two larger helices occurs after the rate-determining transition state for folding as

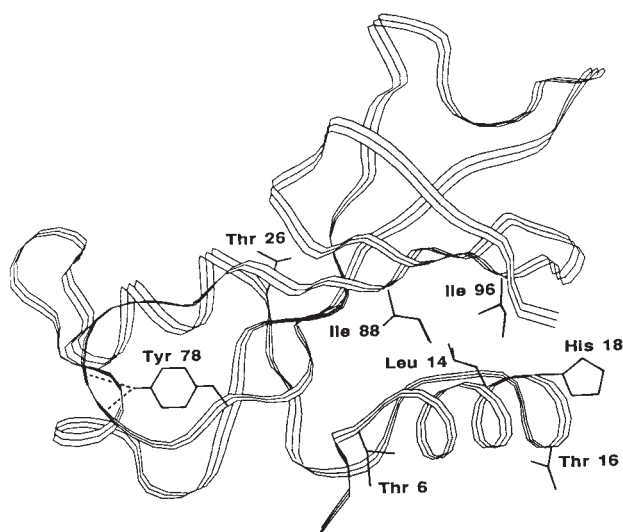


FIG. 5 Sketch of barnase illustrating the target residues.

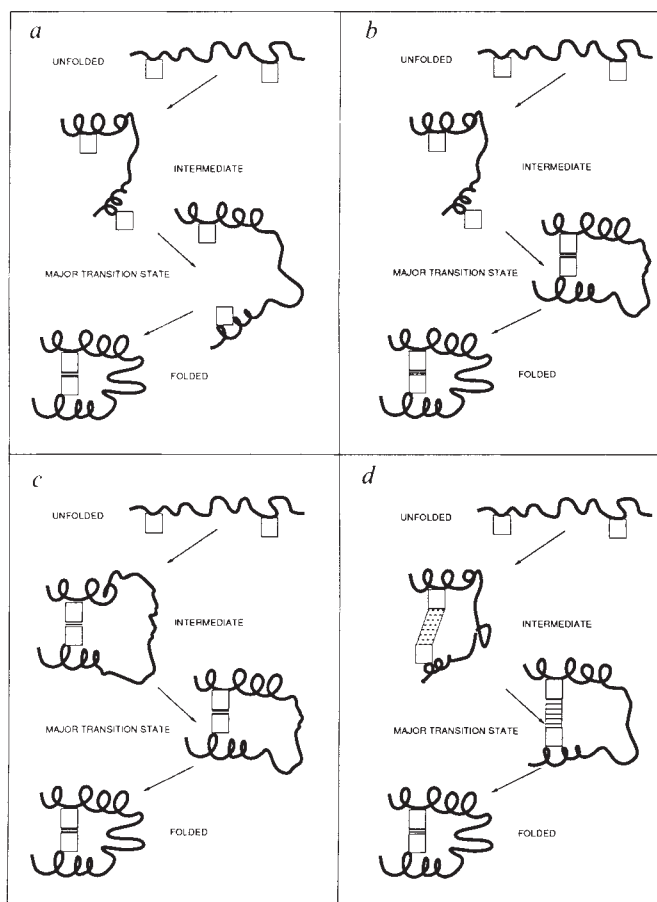


FIG. 6 Possible steps in the folding of barnase. *a*, Two residues do not interact in the unfolded, intermediate or transition states but form after the rate-determining step. Mutation of these does not affect the kinetics of folding but alters the unfolding rate constant. Examples of this are the N-caps and Tyr 78. *b*, The residues interact in the transition state and folded state. No examples yet found. *c*, Interactions occur from the intermediate onwards. Examples approximating to this are His 18 and Thr 16 interacting with residues in the major α -helix. *d*, Progressive bond formation, as with the hydrophobic core.

does the closing of the loop containing Tyr 78. The hydrophobic core between the 6–18 helix and the antiparallel β -sheet consolidates as folding proceeds, implying that the antiparallel β -sheet must be present to some extent. Much of the structural detail of the intermediate is the same as that deduced earlier for the transition state¹², the most striking difference between the intermediate and transition state discovered so far being the weaker

hydrophobic core. The weak hydrophobic interactions in the intermediate may be due either to the core being fluid and weakly packed or to the intermediate being composed of an equilibrium mixture of many states, some of which are well packed and others not. In either case, formation of the final folded structure from the intermediate requires the precise docking of elements of the preformed secondary structure to give a closely packed core and intact loops. Interestingly, the interactions studied here, apart from those in the core, are either close to being fully formed or not formed at all in the transition state or intermediate (Fig. 6). NMR-H-exchange¹³ confirms the existence of the secondary structure and shows that the C termini of all three α -helices are formed in the intermediate as are many of the interactions in the sheet.

Earlier work on stable molten globule intermediates showed general evidence for secondary structure and qualitative evidence for a weakened hydrophobic core^{1–9}. This description fits the intermediate found on the folding pathway of barnase. But NMR studies on the molten globule state of α -lactalbumin, which has extensive secondary structure and a condensed hydrophobic core, show only a few NH groups that are significantly protected against exchange^{2,9} whereas the NH groups in barnase are extensively protected. The protein engineering methods suggest changes that can be made to stabilize the intermediate in barnase so that it can be studied directly by NMR. Certain mutations, such as those in the loops and N-caps, destabilize the folded structure but not the intermediate. Judicious choice of a combination of these mutations should destabilize the native folded state to such an extent that the intermediate should become the predominant compact state at low concentrations of urea and so may be studied directly. Kinetic studies on further mutants should enable us to map most of the structure of the intermediate as well as that of the transition state¹².

Our results show the strengths and limitations of the NMR and protein engineering methods, which are in many ways complementary. NMR gives detailed information about the extent and timing of formation of secondary structure. Protein engineering gives detailed information about the extent, timing and strengths of formation of tertiary interactions, and indirect evidence about secondary structure. Protein engineering is more general as most side chains can be readily modified, and it can be applied to proteins which are too large to be examined by NMR. Kinetics is the only method that can be used to study transition states. Both methods are indirect, as interactions are deduced rather than observed directly. But the consistency of results between the two methods implies that they are giving correct information.

One approach to predicting tertiary protein structure is to predict the repeating secondary structures, such as α -helices and β -sheets, and then dock them (see ref. 23). Our results suggest that docking of preformed elements is indeed part of the rate-determining process in folding and provides encouragement for that theoretical approach. □

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Understanding recent observations of the large-scale structure of the universe

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RECENT galaxy redshift surveys¹⁻³ offer evidence of a quasi-periodic network of 'sheets', with typical dimensions of $\sim 100h^{-1} \text{ Mpc}$ (h is the Hubble constant in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$). Galaxy formation models in which large-scale structure forms purely by gravitational instability from small initial irregularities seem to have problems producing structures on such large scales without violating constraints on the anisotropy of the microwave background, unless they start from apparently contrived initial-fluctuation spectra. Models in which galaxies and clusters have a non-gravitational (possibly explosive) origin seem to be better candidates for the progenitors of large sheets and bubbles, but one would still not expect a perfectly regular lattice. So how surprising are the observations, if they are interpreted in the framework of such models? Here I show that a random cellular model, which mimics the galaxy distribution formed as matter is swept up into shells around expanding void regions or by cosmic blast waves, reproduces the observed Great Walls, voids and periodic structures without undue difficulty.

The most exciting recent developments in observational cosmology have been the results obtained from the surveys of galaxy redshifts¹⁻³. Unfortunately, only partial information about the true three-dimensional distribution of galaxies is obtained from such surveys because of difficulties in obtaining a complete sample. Wide-angle surveys can only reach out to relatively low redshifts so attempts to study the distribution out to much higher redshifts must use a thin almost one-dimensional, sample volume (for example, see ref. 3). Results from the Center for Astrophysics (CfA) survey^{1,2} give the impression of a rather 'bubbly' structure or possibly a pattern of intersecting 'sheets' in the planes of which most galaxies lie. Because of the relatively short range of this wide-angle survey, one cannot see many of these structures and their significance is difficult to assess, although they have typical sizes of at least several tens of megaparsecs. The remarkable results of Broadhurst *et al.*³ seem to confirm the sheet structure because they find a very highly clustered distribution of galaxies along a single line of sight through space, qualitatively consistent with what one would expect to see if one drilled through a bubbly pattern. What is remarkable, however, is the regular spacing of the clumps along the line of sight (to $\sim 10\%$ of the mean spacing). Given that virtually all models of galaxy formation involve some sort of random initial conditions, it is very difficult to see how any such regular pattern could have evolved. It is therefore important to see if any reasonable stochastic clustering models can reproduce the observations.

One of the simplest methods of generating stochastic cellular patterns is to use a geometric tessellation model. The most useful planar or volumetric tessellation is the Voronoi tessellation^{4,5}, sometimes called the Dirichlet or Thiessen tessellation. In three dimensions the Voronoi tessellation consists of space-filling network of Voronoi cells, each of which is a convex polyhedron. A cell is defined by its nucleus and contains the region of space that is nearer to its own nucleus than to any other. The simplest version of the Voronoi model, which I use here, is based on a random spatial distribution of nuclei, that is, the nuclei form a spatial Poisson point pattern, characterized by a mean number density n . Figure 1 shows a two-dimensional Voronoi tessellation. Properties of the tessellation in three dimensions are considerably more complicated than those of the two-dimensional analogue (for example, the mean number of vertices of a two-

dimensional Voronoi cell is just 6, whereas a three-dimensional Voronoi cell has 27.07). Various authors⁶⁻¹⁰ have studied this and related models, concluding that: (1) if galaxies populate the faces of the Voronoi cells, then the model reproduces the qualitative pattern of cells and sheets found in the observations; (2) if rich clusters of galaxies form at vertices of the tessellation, where more than two cells meet—this is where very high overdensities would be expected to form—then the observed correlation function of rich Abell clusters can be reproduced⁸ if $n \approx (100h^{-1} \text{ Mpc})^{-3}$; (3) there is strong physical motivation for the Voronoi tessellation as an approximation to the matter distribution formed at late times in many physical models, whether the driving force is provided by explosions centred on the Voronoi nuclei or by gravitational expansion of underdensities (in the latter case, the Voronoi nuclei represent peaks of the initial peculiar gravitational potential and the tessellation thus formed traces out the large-scale pattern produced by N -body evolution to a remarkable accuracy⁶. None of these conclusions is drastically altered if the Voronoi nuclei are moderately clustered^{7,8}, showing that the applicability of the construction is not dependent on a particular choice of initial conditions, so the results I obtain for the simple Poisson version of the model are quite general. Of course, some galaxies will form outside the cell surfaces, or may move off them once they have formed, so the model can tell us very little about the distribution of galaxies within or in the immediate vicinity of a 'sheet'. One can regard the Voronoi tessellation as a good model for the spatial distribution of the sheets themselves, however, and therefore as an important complement to traditional N -body techniques, which inevitably suffer from problems of sampling and boundary effects on such large scales.

Despite its apparent simplicity and its extensive use (for example, in stereology¹¹) many of the geometric properties of the Voronoi tessellation were not known analytically until recently. Møller¹² has derived some important new results concerning the Voronoi tessellation and has investigated the properties of two-dimensional slices and one-dimensional lines of sight through a three-dimensional Voronoi foam. Without giving the mathematical derivation, I demonstrate below the importance of these results for the interpretation of clustering data.

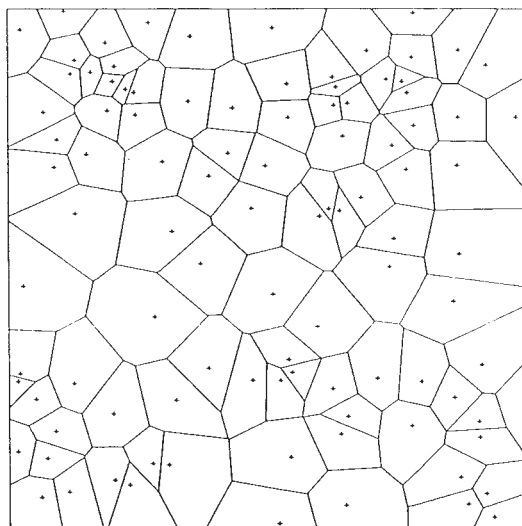


FIG. 1 An example of a two-dimensional Voronoi tessellation generated by a random spatial distribution of 100 nuclei. Although the tessellation is generated by a totally random point set, it exhibits a surprising degree of regularity, particularly in the sizes of the larger cells. This regularity is even more pronounced in three-dimensional tessellations and would be enhanced even further by the destruction of small cells by nonlinear dynamics.

First I consider a line of sight through a Voronoi foam. Such a line will intersect with faces of different cells at (random) spacings λ_1 . Møller has shown that, regardless of the distribution of nuclei

$$\langle \lambda_1 \rangle = 4 \langle V \rangle / \langle A \rangle \quad (1)$$

where V and A are the volume and surface area of a randomly selected cell, and the angle brackets denote expectation values). In the simple Poisson model, this can be calculated exactly

$$\langle \lambda_1 \rangle = \frac{(81/4n\pi)^{1/3}}{\Gamma(2/3)} \quad (2)$$

where $\Gamma(x)$ is the Gamma function. Using the favoured value of n , $(100h^{-1} \text{ Mpc})^{-3}$, leads to $\langle \lambda_1 \rangle \approx 137h^{-1} \text{ Mpc}$, remarkably close to the spacing of clumps observed by Broadhurst *et al.*, $\lambda \approx 128h^{-1} \text{ Mpc}$; the disparity between these values is well within the uncertainty introduced by using Abell cluster correlations to fix n (ref. 8). Of course, the mean clump spacing is not the whole story. The Broadhurst *et al.* data display a set of eight or nine spacings with a dispersion less than 10% of the mean. Is this surprising? Using a very weakly clustered null model, they suggested that the probability of such an occurrence is $\sim 3 \times 10^{-4}$. Such problems are very difficult to solve rigorously, for two reasons. First, various complicated selection effects are involved: our Galaxy is presumably on a cell wall in this model; the four other fields in the original study of Broadhurst *et al.*¹³ do not show such a regular pattern, imposing an *a posteriori* selection on the field that exhibits the structure; the line of sight goes through the Coma supercluster which is known to be a region of extremely high density; neighbouring Voronoi cell volumes are not independent; the smaller cells would be erased by nonlinear dynamics. The second problem is that higher-order moments of the properties of Voronoi cells are not known and these are required to construct a significance test. Møller does, however, give results for the moments of volumes of cells of a related tessellation, called the Delaunay tessellation, which is known to have similar sampling properties to the Voronoi. Scaling the moments of this distribution to the mean Voronoi cell volume gives an estimate of the moments of the distribution of Voronoi cell volumes. The probability that a sample of nine cells leads to a set of line-of-sight intersections with standard deviation $< 10\%$ of the mean can now be calculated. It is a well-known result (see, for example, ref. 14) that the variance of the variance of a sample of n observations drawn from a population with k -th order central moments μ_k is $(\mu_4 - \mu_2^2)/n$. Using the estimates of the moments μ outlined above, I can use this to give a rough estimate of the probability of obtaining a sample variance as small as that observed from nine cells. The result, admittedly very approximate, is $\sim 5\%$, much higher than the result quoted by Broadhurst *et al.*³ which was due to the lack of any large-scale clustering in the null model they used.³ It is clear that the selection effects outlined above will ensure that this result is a rough lower bound on the actual probability of reproducing the observations in ref. 3, so I conclude that if one uses a test model that reproduces the known cluster correlation function, the significance of the observed structures decreases substantially. Also only one out of five independent fields in the study¹³ showed this marked regularity; this may be indicative of a probability of the order of that calculated above. The low significance level can be understood visually by looking at the apparent regularity of the tessellation in Fig. 1: this regularity in cell size increases with the dimensionality of the tessellation. Of course, the only way to simulate the observations reliably is with a Monte Carlo technique: such a study is in progress and seems to be giving results in broad agreement with the rough estimates given here (V. Icke, personal communication).

One can also use Møller's results to look at the properties of the CfA data^{1,2}. The most striking feature of these results is the

presence of a 'great wall' with dimensions $\sim 20 \times 60 \times 100h^{-1} \text{ Mpc}$. In the Voronoi model, this would be interpreted as a face of a Voronoi cell, with the smallest dimension being the thickness of the face caused by the motion of matter out of the plane defined by colliding shock fronts. The mean area of such a face F is

$$\langle F \rangle = \frac{35(2^8\pi)^{1/3}\Gamma(2/3)}{(9n)^{2/3}(24\pi^2 + 35)} \quad (3)$$

Using the favoured value of n , I calculate $\langle F \rangle \approx 3,800(h^{-1} \text{ Mpc})^2$, rather smaller than the $6,000(h^{-1} \text{ Mpc})^2$ for the Great Wall. But this is not too difficult to explain—as the wall is appreciably curved, it may be two or more adjacent faces of the tessellation.

Next, I consider 'slices' such as the preliminary CfA survey¹, which comprised a roughly two-dimensional section through the spatial distribution. This data has a bubbly appearance and includes a void region with diameter $\sim 50h^{-1} \text{ Mpc}$. The mean area of a cell of the two-dimensional tessellation obtained by slicing a three-dimensional Voronoi tessellation is

$$\langle S \rangle = \frac{15}{\Gamma(1/3)(16\pi^5 n^2/9)^{1/3}} \quad (4)$$

giving $\approx 6,860(h^{-1} \text{ Mpc})^2$, which is roughly comparable to the Bootes Void¹⁵ and bigger than the voids seen in ref. 1.

I therefore conclude that the features observed in galaxy redshift surveys are not particularly improbable if the large-scale galaxy distribution follows a simple Voronoi tessellation. At first sight, one might think that the most obvious way of producing such a large-scale pattern physically is in an explosion model¹⁰, but it provides a reasonable approximation to the distribution of matter on large scales even for gravitational instability models such as cold dark matter^{16,17}: most theoretical calculations of large-scale clustering take account only of the linear growth of overdensities and, as argued by Icke¹⁸, it may be the underdensities that are the dynamically important factor—after all, most of the Universe is actually in the voids⁷. The simulations in refs 16 and 17 seem to produce structures similar to those reported¹⁻³ and void expansion may well be the reason for this, although the microwave background places severe constraints¹⁹. Further theoretical work is needed. We need to know how many lines of sight produce a regular pattern like the one observed. The angular size of a typical Voronoi cell face at $z = 0.2$ is $\sim 1^\circ$, so a pencil beam aimed only a few degrees away from the one that produced the regular spacing should produce a much less regular pattern of intersections. Until this is shown not to be the case, the data can be said to fit fairly comfortably within a physically plausible model. $\square$

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Spatial structures in microtubular solutions requiring a sustained energy source

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MICROTUBULES are believed to be the principal organizers of the cell interior¹. Cells respond to a variety of stimuli by modifying the spatial distribution of the microtubules. These effects are central to cell division and morphogenesis², and embryo development³. During embryo development, macroscopic patterns are frequently observed³. Here we report that microtubular solutions spontaneously form alternating white and dark stripes about 1 mm wide and 1 cm long. Small-angle neutron scattering measurements show that in each segment the microtubules are aligned obliquely to the direction of the stripe, and that the white and dark stripes differ in having mutually orthogonal orientations. The formation of these structures requires an initial reservoir of organic phosphate. Phosphorus NMR measurements show that the process is accompanied by the energy-liberating conversion of organic to inorganic phosphate. These observations, together with similarities to the dissipative spatial structure formed by the Belousov-Zhabotinski reaction⁴⁻⁶, provide strong evidence that the observed structures are energy-dissipative in nature. Dissipative structures are thought to be critical to the appearance of complex living organisms^{7,8}. Our results strongly suggest that microtubules are capable of forming such structures. Microtubular dissipative structures may occur during mitosis and embryo morphogenesis.

Tubulin was purified from recycled bovine brain microtubule protein using standard phosphocellulose chromatography⁹. This preparation is known not to contain any significant amounts of impurities. We dissolved the tubulin in heavy water (D₂O) so as to obtain an adequate neutron scattering signal. Microtubules were formed by warming the tubulin (3–20 mg ml⁻¹) from 4 °C to 33 °C in the presence of 1 mM GTP, 20 mM acetyl phosphate and acetate kinase (1.25 × 10⁻³ mg ml⁻¹); their formation, monitored by turbidity and neutron scattering, was completed after ~15 min. At this stage, the samples appear uniform but over a period of 3–10 h, a series of horizontal white and dark stripes develop, which gradually encompass the whole cell (Fig. 1). The stripes are strongly birefringent. Once formed the structure remains stable for >24 h. On cooling to 4 °C the stripes disappear and the sample becomes transparent. On rewarming to 33 °C they reappear. These observations, together with the neutron scattering results discussed below, show that the stripes arise from microtubules. They are best viewed in reflected light, with the light coming to the cell obliquely (45°). Turning the cell by 90° interchanges the white and dark regions and rotating the cell by 45° minimizes the contrast. The samples were contained in quartz cells with optical path lengths of 1 or 2 mm; patterns of the same type were observed in cells of path length 0.5–5 mm.

We used small-angle neutron scattering¹⁰ to investigate the microtubular structure of the stripes. Figure 2 shows contour plots of the neutron counts detected with a 64 × 64 two-dimensional array. The distribution of scattered intensity is highly asymmetric with most of the intensity concentrated into an arc, oriented at ~45° or 135° to the horizontal for the dark and white stripes, respectively. Plots of scattered intensity taken along these arcs show the scattering profile of microtubules^{11,12} as viewed along their cross-section, and the spectra from the different stripes are of equal intensity. These results show that both stripes arise from equal concentrations of microtubules; the fact that the scattering is concentrated into an arc, and not

a circle, indicates that the microtubules are aligned with their long axis perpendicular to the direction of scattering. The stripes are therefore regions of microtubules, alternately oriented at ~45° and 135° to the horizontal. This was confirmed by the scattering pattern using a vertical slit covering several stripes, which showed arcs at both 45° and 135°, consistent with the sum of the spectra from both orientations. The spots in Fig. 2 are not symmetrical, but show a trail towards the horizontal, consistent with a preferred misalignment of the tubes towards the vertical. This is the behaviour expected at the interface between stripes, where tubes of a 45° orientation form an interface with tubes of a 135° orientation. The microtubules in each segment showed no positional ordering between themselves, hence each stripe constitutes an orientated nematic liquid-crystalline phase.

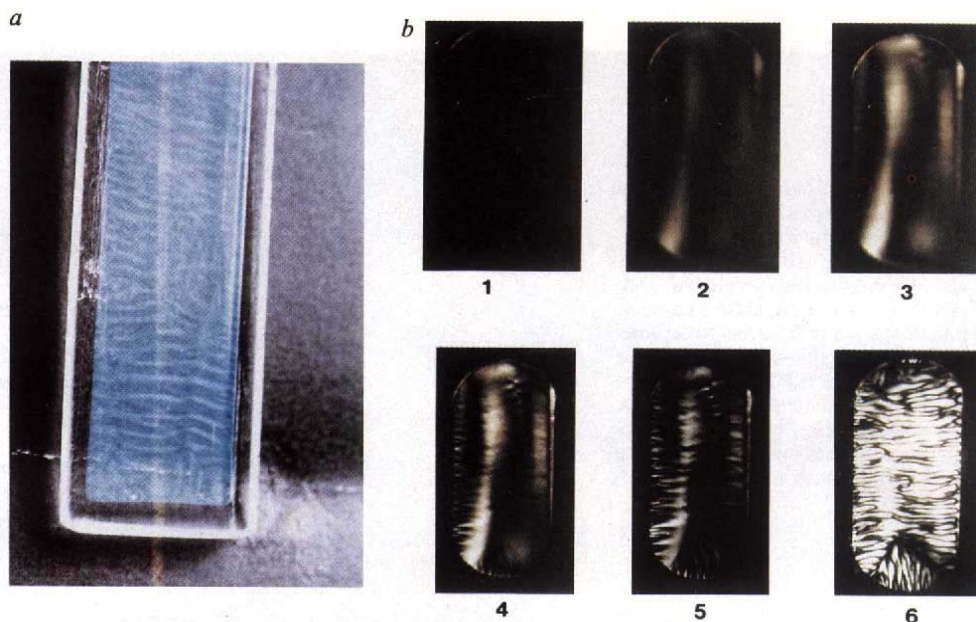
This structure also explains the optical observations, because the tubules will strongly scatter light when viewed down their length, but will only scatter weakly, and therefore appear dark, when viewed across their diameter. Thus, the contrast is best when illuminated obliquely, and the light and dark regions are interchanged when the sample is rotated by 90°. Moreover, a stripe that is white when illuminated by light at 45° should show neutron scattering spots, arising from the microtubular cross-section, at 135° to the horizontal, as is observed. Also because the microtubular orientations are 45° and 135°, the stripes observed through crossed polarizing filters, at 0° and 90°, are of uniform birefringence, except for a line of extinction where the orientation changes. This corresponds to the observations shown in Fig. 1b.

Above a critical concentration, anisotropic objects such as long rods may spontaneously orient as a result of packing considerations¹³. Although this might explain the orientational ordering of the microtubules in each stripe, it does not account for the alternating mutually orthogonal orientations. The spontaneous formation of macroscopic periodic ordering is a property of nonlinear systems that are far from equilibrium^{4-8,14-18}. Formation of these structures requires continual energy consumption or dissipation to drive the system and maintain it in a state of higher order and lower entropy than that arising under equilibrium conditions. The energy may be provided internally, by a chemical reaction such as in the Belousov-Zhabotinski reaction or externally, as in the application of a temperature gradient to a liquid layer (Bénard instability)^{6,7,15}, or the application of electric and magnetic fields to liquid-crystal films^{19,20}. Under certain conditions, systems of the first type can generate chemical oscillations in time. Such oscillating chemical reactions have been observed for microtubules²¹⁻²³. Microtubule formation and stability require the continual energy-liberating hydrolysis of GTP to GDP²³⁻²⁵. The overall process is thermodynamically irreversible²⁴ and is thought to be nonlinear^{22,23}. Microtubules are thus associated with the properties required for the formation of dissipative structures.

The requirements for a structure to be termed dissipative are that the system be thermodynamically irreversible with a net input of energy, and that there be an energy source for the formation and maintenance of the structure. To provide a continuous source of GTP at constant concentration, it is common practice to add a solution of acetyl phosphate together with a GTP regenerating enzyme²⁶. The formation and maintenance of the microtubules results in a net reaction in which acetyl phosphate is gradually converted into inorganic phosphate.

To provide direct evidence that the same process occurs in this system we took ³¹P NMR spectra at different times during and after the formation of the striped structures. Spectra taken at different times are shown in Fig. 3. The three low-intensity peaks on the right arise from GTP and the two resonances on the left arise from inorganic phosphate and acetyl phosphate. With increasing time, the peak arising from inorganic phosphate increases at the expense of that from acetyl phosphate. Except

FIG. 1 *a*, Striped patterns formed by microtubular solutions at 33 °C observed in reflected light. The sample cell dimensions were 40 × 10 × 1 mm. The sample comprised tubulin (10 mg ml⁻¹) and GTP (1 mM) in a D₂O buffer containing 100 mM MES (2-*N*-morpholino ethanesulphonic acid), 1 mM EGTA (ethylene glycol-bis-(*B*-aminoethyl ether) *N,N,N',N'* tetra-acetic acid), and 1 mM MgCl₂ at pH 6.75. Microtubules continually dissipate energy by transforming GTP (guanosine triphosphate) to GDP (guanosine diphosphate). A regenerating system of acetyl phosphate (20 mM) and acetate kinase (1.25 × 10⁻³ mg ml⁻¹) was added to provide a continuous source of GTP. *b*, Microtubular solution, observed through crossed polarizing filters, at different times after initiating microtubule formation by warming from 4 °C to 33 °C; 1, 1 min; 2, 20 min; 3, 2 h; 4, 3 h; 5, 4 h; 6, 13 h. The sample composition was the same as in *a* except that the tubulin concentration was 4.6 mg ml⁻¹, and the acetate kinase concentration was 5 × 10⁻⁴ mg ml⁻¹. The optical path length was 2 mm. The length of the microtubules during and after pattern formation was determined, using known procedures²¹ to be ~5 µm. These patterns have been reproduced many times in different



locations and environments, indicating that they do not arise from uncontrolled external factors such as vibrations. The appearance of the microtubular stripes shows a strong resemblance to the establishment of the dissipative space structure in the Belousov-Zhabotinski reaction.

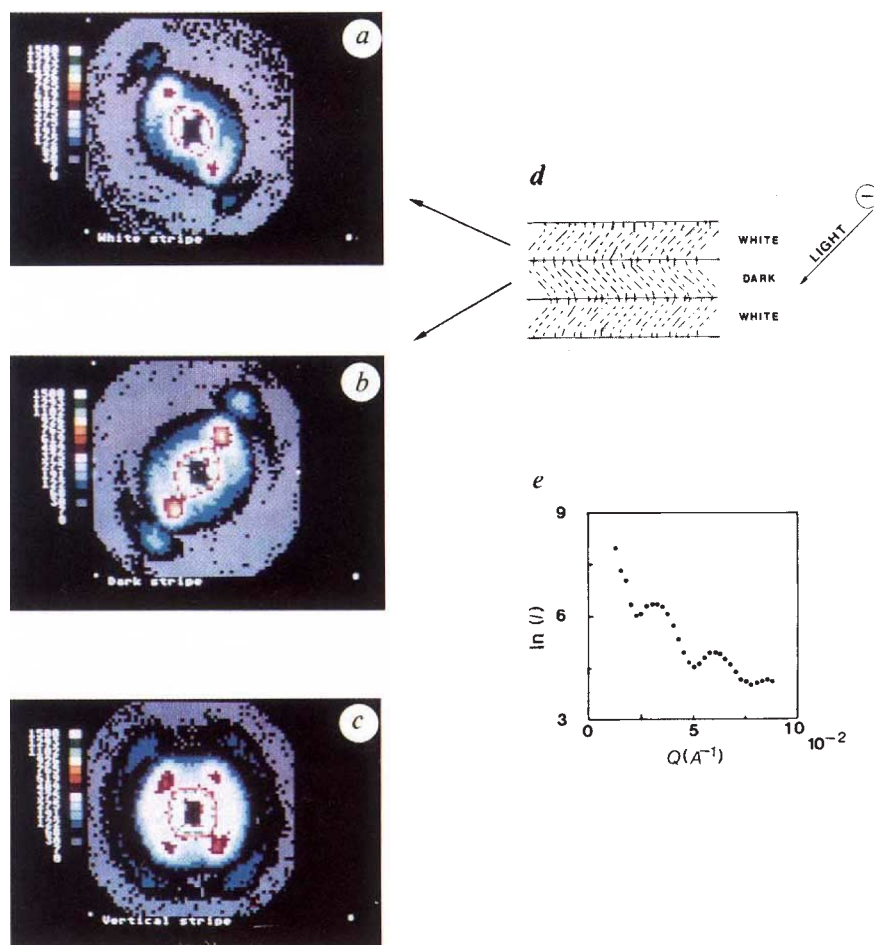
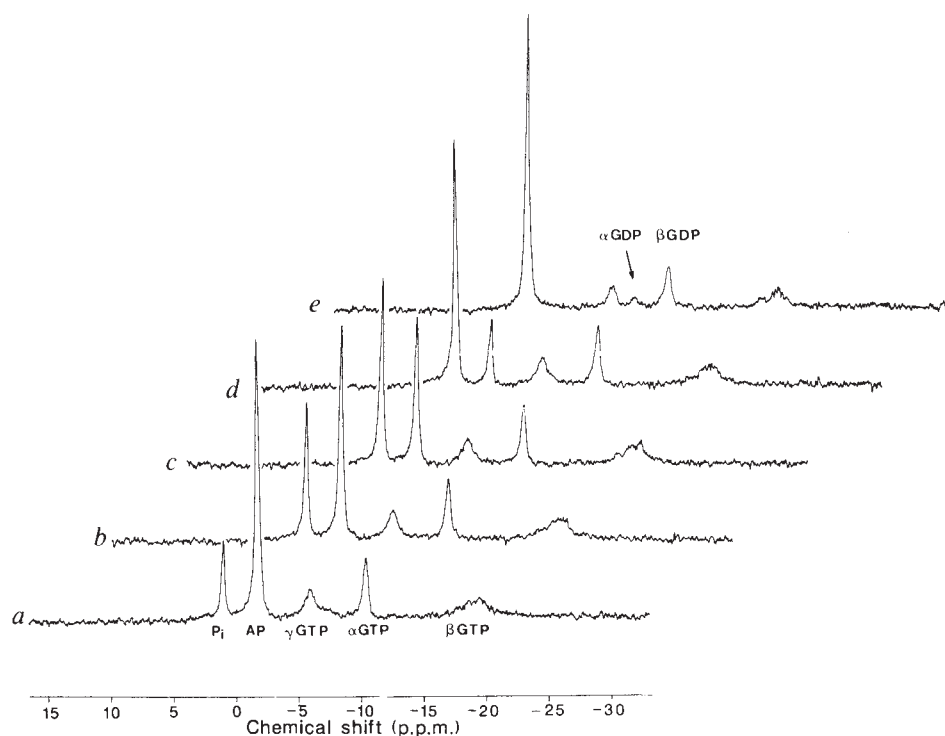


FIG. 2 Neutron scattering spectra from *a*, a white stripe, *b* a dark stripe, *c* a vertical slit across several stripes (same sample as in Fig. 1*a*, top left corner). The different neutron-bombarded regions were pre-selected using a cadmium mask with a slit (½ × 6 mm) at different places on the front of the sample cell. Spectra were measured on the DII camera³² (Institut Laue-Langevin) using a 2.5-m sample-detector distance and neutrons of 10-Å wavelength. Counting times were ~2 h. The results are shown as contour plots of the number of scattered neutrons on a 64 × 64 two-dimensional detector. The neutron beam, stopped by a rectangular cadmium plate after transmission through the sample, is centred on the detector. *d*, Schematic representation of the periodic microtubular structure formed. The microtubules are represented as dark rods. *e*, Scattering profile taken along one of the arcs, corresponding to the spectrum from microtubules^{11,12} viewed along their cross-section. The momentum transfer Q is defined as $4\pi/\lambda \sin \theta$ where λ is the neutron wavelength and 2θ the scattering angle.

FIG. 3 ^{31}P (162 MHz) NMR spectra measured at different times during and after the formation of the dissipative structure: a, 40 min; b, 100 min; c, 200 min; d, 400 min; e, 24 h. The sample was contained in a 15-mm tube without spinning. Individual spectra took 13 min to accumulate. Chemical shifts are relative to an external sample of H_3PO_4 . The resonance marked P_i and AP arise from inorganic phosphate and acetyl phosphate respectively. The position of the β -GDP resonance coincides with that of α -GTP.



for the last spectrum, the intensity of the GTP signals remains the same, signifying a constant concentration. When the acetyl phosphate is exhausted, the acetate kinase can no longer recycle the GDP, and after ~ 24 h a signal from GDP starts to appear. At this stage the kinetics are very slow, and the striped aspect and birefringence of the sample slowly begin to disappear; this is paralleled by a decrease in the total mass of polymerized tubulin until complete denaturation of the sample occurs.

The spectra shown in Fig. 3 confirm the reaction scheme outlined above and show that the formation of the striped structure is accompanied by an irreversible chemical reaction. As the hydrolysis of acetyl phosphate to inorganic phosphate is exothermic, energy is dissipated into the system. To show that this energy is essential to the formation and maintenance of the structures we prepared a series of samples containing 1 mM GTP and of acetyl phosphate concentrations varying from 0 to 20 mM. With 1 mM GTP and no acetyl phosphate, no stripes were formed. Stripes begin to form and become more marked as the acetyl phosphate concentration is increased. Turbidity measurements showed that, to within 10%, the same mass of microtubules were formed for all the samples. Also with increasing acetyl phosphate concentration, the structures were maintained for progressively longer periods. These results show that the acetyl phosphate is necessary for the formation and maintenance of the structures and at the same time constitutes an energy reservoir which is irreversibly consumed during the process.

The NMR results show that the rate of acetyl phosphate consumption is much higher during the formation of the stripes than after. Experiments of this kind should therefore permit the separation of the energy required for structure formation and structure maintenance. Raising the concentration of magnesium in the buffer from 1 to 10 mM increased the rate of energy dissipation, and the sample no longer showed the striped structure.

The appearance of the microtubular stripes shows a striking resemblance to the formation of layers in the dissipative space structure of the Belousov-Zhabotinski reaction⁶. Also, long rod-like polymer liquid crystals that are far from equilibrium owing to the application of an external magnetic field form a similar dissipative structure comprising alternating layers of different

orientation²⁷. Together, these observations strongly suggest that the microtubular state reported here is a dissipative spatial structure explicable in terms of irreversible nonlinear processes.

While this work was being refereed, two related articles have appeared. Mandelkow *et al.*²⁸ have demonstrated dissipative behaviour in a sample showing bulk oscillations of microtubule assembly and disassembly. Depending on the conditions, dissipative systems can show either time-dependent or steady-state structures. The behaviour reported by Mandelkow seems to be of the time-dependent kind, whereas here a steady-state structure is achieved. Hitt *et al.*²⁹ have reported optical observations of a macroscopic microtubular structure similar to ours. They did not detect the periodic mutually orthogonal microtubule ordering from stripe to stripe, however, and offered no explanation of the striped structure. They realized that the microtubules were oriented by domains and explained the local orientational ordering in terms of packing considerations. But, as mentioned earlier, such considerations do not account for the striped structure nor its dependence on an energy source. Their samples were made in H_2O buffers, and therefore the dissipative structures are not restricted to D_2O -based buffers and could arise *in vivo*.

Mitotic spindles, which are constituted largely of microtubules show a pattern of bright and dark regions when viewed through a polarizing microscope^{24,30}, consistent with the spindles containing orthogonally oriented microtubular domains. The stripes reported here show substructures similar to those of mitotic spindles²⁴. This aspect of our observations will be discussed elsewhere, but suggests that microtubular dissipative structures may form during mitosis. □

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Differential interaction of chiral β -particles with enantiomers

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VESTER¹ and Ulbricht² invoked the parity-violating weak interaction as the cause of biological homochirality. They postulated that a stereoselective synthesis or decay of chiral molecules could be induced by the left circularly polarized bremsstrahlung; this small effect would then be amplified to full homochirality^{3–5}. Despite much effort during the past thirty years, experimental results have been inconclusive and contradictory^{6–12}. The aim of the study described here was more modest; we did not want to demonstrate differential synthesis or the decay of enantiomers bombarded by β -particles. Rather, using pulse-height spectroscopy, we examined the Cerenkov radiation emitted as β -particles pass through liquid chiral enantiomers. Our results show that helical electrons do distinguish between molecules of opposite chirality, and complement results reported in earlier publications in which differential decay^{6,13}, positron annihilation^{14,15}, scattering^{16,17} or other interactions^{18–20} were observed.

The expectation of differential interaction of helical β -particles with enantiomers is based on an analogy with the theory of optical rotary dispersion (ORD) and circular dichroism (CD). There, the refractive indices and molar absorption coefficients of enantiomeric pairs are slightly different for left and right circularly polarized photons owing to the opposite helical potential field provided by the helically distributed static charges of the enantiomers. Thus the helical photons propagate with different velocities (ORD) and are absorbed to different extents (CD). Similarly, the left-helical β -electrons, with spin and momentum anti-parallel, should propagate with different velocities in the two enantiomers. The energy exchange between naturally chiral β -particles and the oppositely chiral molecules should also be different. One of us proposed¹² that the differential effect could be measured by pulse-height spectroscopy of the Cerenkov radiation, because this also depends on the refractive indices of the medium. We used enantiomers that are liquid at room temperature to avoid problems associated with dilution in achiral solvents.

Cerenkov radiation is the bluish light emitted as high-energy charged particles pass through a transparent medium at a speed v that is greater than the velocity of light c in that medium^{21,22}. The intensity of Cerenkov radiation, a pulse, is proportional to the energy of the charged particle; a plot of counts per minute (c.p.m.) against channel number yields a pulse-height spec-

trum²³. Provided the magnitude of the effect is large enough, the scintillation counter should detect an energy shift in the pulse-height spectra of β -particles in oppositely chiral molecules, because of the different propagation velocities. But the magnitude of the effect cannot be predicted because the Bethe equation^{24,25}, which relates the stopping power of the medium to the energy of the β -particles, contains neither the chiral form factor for the medium, nor the spin momentum coupling of the β -particles. Preliminary results¹² were promising; the shift was observed, but the resolution of the pulse-height spectra was unsatisfactory. Here we report high-resolution pulse-height spectra which allow us to calculate the energy exchange between β -particles and enantiomers, the slowing time of the β -particles and the stopping powers of the enantiomers.

The resolved enantiomers (R)-(-)- and (S)-(+)-2-phenylbutyric acid (PBA) are liquid at room temperature. Optical rotation in different samples (chiral purity 99+%, Aldrich Chemical Co. and Norse Laboratories) varied between $[\alpha]_D = -88.0$ to -93.0 in case of R-PBA, and $[\alpha]_D = +89.2$ to $+92.0$ for S-PBA. The refractive index and density were 1.5156 and 1.055 g cm⁻³, respectively. Absorption, luminescence, infrared and CD spectra indicated that there was no contamination; spectra were essentially identical for the R- and S-enantiomers. ³²P (carrier free, H₃PO₄; ICN and New England Nuclear) emits a continuum of β -particles with mean and maximum energies of 695 keV and 1,710 keV, respectively; the corresponding left-handed polarizations (v/c) are 0.906 and 0.973, as calculated from the refractive index. The Cerenkov low-energy threshold for PBA is 169 keV, corresponding to 0.690 polarization, thus ~90% of the β -particles will emit Cerenkov radiation. For details of the method, see ref. 12.

Although it was tacitly assumed that the β -particles are emitted from the ³²P nuclides with the same energy in both enantiomers, it cannot be decided *a priori* that the chiral environment does not influence the energy of the β -particles. R- and S-PBA were therefore bombarded with external ³²P β -particles—instead of dissolving the H₃³²PO₄ in the liquid enantiomers, it was applied to and dried on a brass rod, covered with a 0.016-mm-thick sheet of polyvinyl chloride, and positioned in the vial ~1 cm above the liquids.

Figure 1a shows that the pulse-height spectra of R- and S-PBA with dissolved ³²P β -particles are not superimposed—the peak of R-PBA appears 13.5 channels toward lower energy than does the peak of S-PBA, an apparent energy difference of 41.4 keV. The longitudinally polarized β -particles therefore distinguish between the mirror-image molecules. The differential spectrum (Fig. 1b) more clearly demonstrates the shift with the scale of the ordinate magnified. The effect is larger than the 2σ uncertainty. When, the enantiomers are bombarded by β -particles from an external source, however, there was a noticeable decrease in the resolution of the differential spectrum (Fig. 1c). The peaks of R- and S-PBA differ by only six channels and the standard deviation is large. Therefore an influence from the chiral environment, cannot be entirely discounted. These results may, however, reflect the effects of an inherently different geometry of samples bombarded from an external source. The β -particles from an external source collide first with PVC and air molecules, and thus partially lose both velocity and polarization before reaching the PBA molecules. The density of interaction between electrons from the external source and PBA is also greater toward the surface of the enantiomer and interference phenomena may occur. These differences may also explain why previous investigations, in which molecules were bombarded from an external source^{7–9}, did not detect a differential interaction between chiral molecules and β -particles.

The differential spectra produced by polarized ³²P β -particles were compared with those produced by non-polarized high-energy electrons. Pellets of ¹³⁷Cs (Amersham Co.) were enclosed in a β -particle-absorbing shield (made of 2-mm-thick layers of copper and lead) and positioned inside the scintillation vial

TABLE 1 Change in peak position with ^{32}P flux density

Count rate (c.p.m.)				Channel of peak			
R-PBA	Racemic PBA	S-PBA	Water	R-PBA	Racemic PBA	S-PBA	Water
1,343,261 $\pm$ 78,585	1,400,098 $\pm$ 21,056	1,311,497 $\pm$ 47,140	1,050,570 $\pm$ 28,572	288.2 $\pm$ 1.6	299.7 $\pm$ 3.8	299.1 $\pm$ 5.9	262.4 $\pm$ 0.2
791,274 $\pm$ 41,052	844,664 $\pm$ 978	825,198 $\pm$ 26,199	623,518 $\pm$ 16,661	283.6 $\pm$ 3.0	303.1 $\pm$ 2.9	295.9 $\pm$ 6.1	260.3 $\pm$ 0.4
315,592 $\pm$ 6,886	331,845 $\pm$ 645	322,743 $\pm$ 10,328	244,566 $\pm$ 6,358	280.2 $\pm$ 3.8	300.6 $\pm$ 3.4	291.6 $\pm$ 6.3	257.9 $\pm$ 0.6
298,821 $\pm$ 9,199	293,848 $\pm$ 1,910	296,601 $\pm$ 27,906	220,296 $\pm$ 6,649	284.9 $\pm$ 1.6	290.5 $\pm$ 2.2	297.2 $\pm$ 1.2	252.4 $\pm$ 1.3
176,552 $\pm$ 6,500	178,466 $\pm$ 702	176,855 $\pm$ 16,431	132,913 $\pm$ 3,613	283.0 $\pm$ 1.4	300.5 $\pm$ 2.9	296.5 $\pm$ 1.7	256.4 $\pm$ 1.1
68,770 $\pm$ 2,654	70,149 $\pm$ 362	72,605 $\pm$ 6,510	52,193 $\pm$ 1,269	279.5 $\pm$ 1.1	301.5 $\pm$ 4.3	295.6 $\pm$ 1.8	255.7 $\pm$ 2.0

For the Beckman LS 3801 scintillation counter and phenylbutyric acid used in this study, the following equation relates Cerenkov energy (keV) with the channel number, N : $E = \exp[(N + 638.6)/151.7]$. The equation is not accurate for energies ≤ 350 keV owing to the inefficiency of photomultiplier tube at low light intensities. Thus, the pulse-height spectrum does not overlap with the energy spectrum of the β -radiation. Fortunately, the problem is unimportant for our purpose because the peak of the spectrum and the maximum energy of the β -particles from ^{32}P lie far above the 350-keV threshold. The following efficiencies were calculated: R-PBA, 65.3 ± 1.6 ; Racemic-PBA, 66.5 ± 1.4 ; S-PBA, 66.3 ± 2.3 ; water, 49.8 ± 1.8 . The efficiency of water is in agreement with earlier observations²³. The unusually high efficiencies of PBA is due to its high refractive index. The channel of the peaks was determined from a best-fit polynomial to 15 points centred around the maximum of each spectrum; a minimum of four spectra were evaluated for each group. The correlation coefficient was >0.98 for each curve.

* Racemic mixtures were prepared immediately before bombardment but many crystallized during the experiments.

~ 1 cm above the PBA. The γ -particles eject non-polarized Compton electrons from the molecules and these high-energy particles then emit Cerenkov radiation. The differential spectrum of the non-polarized Compton electrons (Fig. 1d) is a straight line, as expected.

Despite the statistical confidence, we conducted two further control experiments to discount the possibility that the observed effect was contributed to or caused by systematic errors associated with the instrument, or by undetected contamination in the PBA. The main concern about the scintillation counter was saturation of the photomultiplier tubes and overspill of signals

from one channel into another. Although experiments with different amounts of ^{32}P demonstrated that high count rates cause a shift of the pulse-height spectra (Table 1), the difference between R- and S-PBA is clearly observable at all count rates, regardless of the shift caused by high count rate, and varies between 11 and 16 channels. The saturation edge is located at $\sim 200,000$ total c.p.m. Table 1 also includes the peak location of racemic mixtures. It was not surprising that the peak of the pulse-height spectra of the racemic mixture is not located between the resolved compounds; the physical parameters of racemic mixtures are different from those of the enantiomers.

FIG. 1 a, Cerenkov pulse-height spectrum of ^{32}P dissolved in R- or S-PBA. Differential spectra (b-f) are generated by subtraction of the count rate in each channel of S from those of R. Differential spectra of b, R- and S-PBA shown in a; c, external ^{32}P and d, Compton electrons from ^{137}Cs . Scintillation differential spectra for e, ^{35}S and f, ^{32}P . Scintillation solutes were PPO and POPOP, see ref. 12 for method. A smaller amplitude for these processes may reflect the multiple achiral energy transfer processes of the liquid scintillation measurement. The first part of the ^{32}P spectra was removed to minimize the Cerenkov effect recorded in low-energy channels and concomitant irradiation from β -particles from unavoidable ^{33}P contamination. The latter may contribute to the high standard deviation on the ^{32}P differential scintillation spectrum. Note the different scales on the ordinate of the differential spectra. All spectra represent the average of four PBA samples measured in random order; the instrument was calibrated before each measurement. Saturation of photomultiplier and overspill between channels were avoided by assuring low count rates; samples of $<200,000$ total c.p.m. were used. All spectra were taken in five-channel increments and normalized to the total count rate. Error bars represent the standard deviation for each point. This set of experiments was repeated eight times with different batches of enantiomers and ^{32}P , a total of 32 spectra. All measurements yielded consistent results.

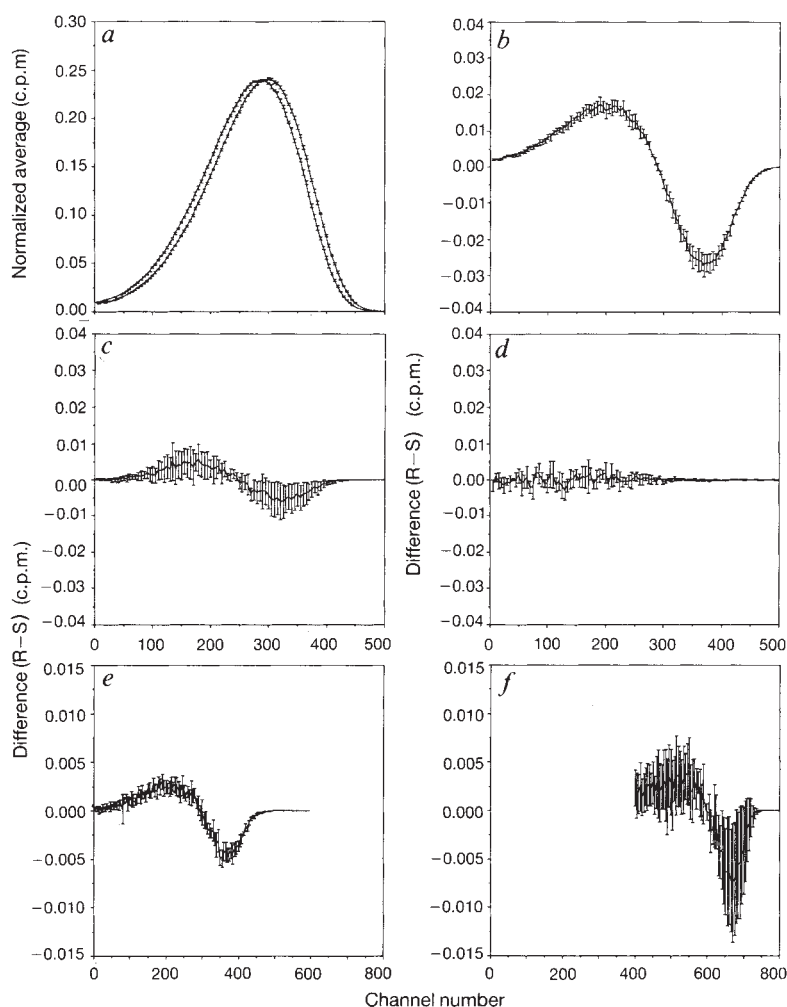


TABLE 2 Cerenkov interaction of β -particles with R- and S-PBA

^{32}P β -interaction	R-PBA	S-PBA
Apparent energy of peak of pulse-height spectrum (keV)	434.50 $\pm$ 4.11	475.50 $\pm$ 5.20
Collisional stopping power ($\text{MeV cm}^2 \text{g}^{-1}$)	2.041 $\pm$ 0.050	1.997 $\pm$ 0.005
Distance travelled (mm)	1.268 $\pm$ 0.017	1.441 $\pm$ 0.022
Slowing down time (ps)	7.939 $\pm$ 0.080	8.731 $\pm$ 0.100

Data obtained by solving the Bethe equation^{24,25}.

Resolved PBA is liquid at room temperature, whereas the freshly made racemic mixture solidifies to soft crystals within a few hours or days. Owing to crystallization, the stopping power of racemic mixtures should not be the median of R- and S-PBA.

To examine the influence of possible contaminants, R- and S-PBA were contaminated with iron ethylenediamine tetraacetic acid; 0.05 μmol is the critical concentration at which the peak of the pulse-height spectra is shifted ~ 7 channels toward lower energy with a concomitant 17% decrease in Cerenkov efficiency. This small concentration is easily detected both spectrophotometrically and visually. As mentioned earlier, absorption spectroscopy did not indicate the presence of colour contamination, nor were efficiency differences between R- and S-PBA observed. A second type of contaminant could be any spectrophotometrically undetectable organic substance such as methanol, ethanol and ethylene glycol, introduced during preparation of the liquid compound. The peak of the pulse-height spectra was at channel 269 to 270 for these compounds. Therefore, a considerable amount of contaminant would be necessary to cause an 11–16 channel shift as observed between R- and S-PBA. In fact, dilution experiments showed that 10% contamination with methanol did not cause a significant change in the pulse-height spectra of either the R- or S-PBA. Such a large contamination is, of course, extremely unlikely; according to the high-performance liquid chromatography data supplied by the manufacturers, contamination of the resolved compounds is $< 0.1\%$.

Table 2 summarizes our calculations. The stopping power of R-PBA for β -particles is significantly higher than that of S-PBA. Consequently, the chiral electrons travel a longer distance and their slowing time is greater in S-PBA than in R-PBA. The analogy between the different refractive indices for circularly polarized light in enantiomers and the differential emission of Cerenkov radiation by helical β -particles is thus supported. Excitation and ionization are the predominant processes by which β -particles lose energy. The Kronig–Kramer^{26,27} theorem relates the refractive index to excitation: from the refractive-index-dependent ORD spectrum the excitation-dependent differential absorption spectrum can be calculated. The extent of the postulated analogy was therefore investigated by the differential scintillation spectrum of β -particles from ^{35}S (carrier free, H_2SO_4 , ICN), the energies of which are below the Cerenkov threshold (mean 49, and maximum 167 keV; $v/c = 0.41$). The scintillation spectrum in Fig. 1e clearly shows a small but significant differential interaction for processes dominated by excitation. The scintillation differential spectrum for ^{32}P is presented for comparison (Fig. 1f).

The Sokolov–Loskutov²⁸ equation addresses the contribution of spin to Cerenkov radiation in achiral media. For the optical properties of PBA and the assumption that the refractive index varies little in the blue-violet region, the calculated spin effect is ~ 1 part in 10^{14} of the refractive-index-dependent emission, an apparently negligible contribution.

The observations reported here complement those in references 14, 15, 17–20, but the mechanism of interaction of high-

energy β -particles in liquid enantiomers and the low-energy ‘artificially’ polarized electrons in chiral gaseous targets¹⁷, must be entirely different.

Finally, the conservation of energy and the cause of the apparent energy difference of β -particles implied by shift of the pulse-height spectra is not fully understood. If the chiral environment influences the energy of the emitted β -particles, then the origin of the shift is easily explained. But if the β -particles are emitted with the same energy in each enantiomer, then the apparently ‘missing’ energy described by the shift reflects a difference in the distribution of the energy loss of the electron. In the S enantiomer, a larger fraction of the dissipation energy would appear as Cerenkov radiation and excitation, and a smaller fraction as translational, rotational and vibrational motion of the molecules; for the R enantiomer, the converse would occur. $\square$

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Evidence for an early glacial maximum in the French Vosges during the last glacial cycle

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THE Last Glacial Maximum is observed to have occurred between 18,000 and 20,000 years ago throughout northern Europe. Here we present evidence for the surprising result that in the region of the French Vosges, the regional ice sheet advanced during the early part of the last glacial cycle, and this expansion was considerably larger than that which occurred during the Last Glacial Maximum. From these results, we conclude that the glacial maximum may not have occurred at the same time worldwide, contrary to what has usually been assumed¹.

Three glaciations successively built up the Linxert moraine complex, the group of Ecomagny terminal moraines and the Servance terminal moraines² (Fig. 1). The ice originated in the eastern Vosges highlands (1,424 m above sea level (a.s.l.)), descending west and southwest through several valleys, the

largest of which is the Moselle. During the first two advances the ice overflowed the northern crest of the Ecromagny Plateau (750–800 m a.s.l.), reaching down to elevations of 300 and 500 m a.s.l. During the Servance glacial advance, however, the ices did not reach the plateau but entered the Ognon valley through the pass at Col des Croix (Fig. 1) which lies at 683 m a.s.l.

All three moraine groups are complex features and may represent several recessions and readvances. The petrographic composition of glacial deposits reflects the bedrock over which the ice advanced—near Ecromagny and Linxert the moraines, tills and related outwash deposits contain a high percentage of detritus from the metamorphic rocks outcropping in the Ecromagny Plateau, whereas the outwash and tills connected with the Servance moraines are dominated by granitic detritus (Fig. 1).

Numerous depressions, which today are marshes and peat bogs, were formed by glacier erosion. Many of them are closed basins without surface outflow, surrounded by higher ground built in part by the bedrock and in part by the tills. Here we discuss cores from two of them—the Grande Pile bog, which lies between the Linxert and Ecromagny moraine lines, and the Cusenier peat bog, which lies on the Plateau behind the innermost Ecromagny moraine (Fig. 1).

Multiple bores drilled in the Grande Pile bog recovered ~18 m of silty clay, peat and gyttja, overlying the glaciolacustrine tills of the Linxert glaciation. In Fig. 2 we show the principal features of the Grande Pile core in a composite section from Woillard^{3,4} to which we have added the ¹⁴C data of Woillard and Mook⁵ and our petrographic information. The pollen

diagram^{3,4} proves that the Linxert glacial advance is older than the last interglacial (Lure) and that the Ecromagny advance, which did not overrun the Grande Pile site, is younger. Three ash layers are present in the Grande Pile core. The Ternuay ash, the source of which is unknown, is older than 70,000 years and its position relative to the pollen zones places it at the end of the Lure interglacial, which is correlated with the oxygen isotope stage 5e (ref. 4). The Melay ash, probably from the Eifel Massif, is between 20,120 and 28,880 years old and the pollen analysis places it in the period of cooling that led to the Last Glacial Maximum. It is correlated with the lower part of oxygen stage 2. The youngest ash is correlated with the Laacher See Tuff 5-Final, which is about 10,800 years old⁶.

The Grande Pile peat bog has always been isolated from the surface hydrographic network and the only contribution of detritic minerals to the predominantly organogenic sediments comes from the local hillwash and from the wind-blown dust or volcanic ash.

We identified the heavy minerals using a petrographic microscope, and verified them using Gandolfi camera X-ray analysis. The heavy minerals are derived either from the granites, metamorphic rocks or Triassic sediments outcropping in the Vosges (Figs 1 and 2). Detritic calcite accompanies the metamorphic group. In the organic muds of Lure interglacial the content of heavy minerals was too low to allow interpretation. The oldest detrital Linxert sediments in Grande Pile originated mostly from sedimentary and metamorphic bedrock. In the Mélisey I zone the granitic assemblage indicates transport from the braided plains of rivers flowing through the granites. In Mélisey II the granitic assemblage is succeeded by the metamorphic one,

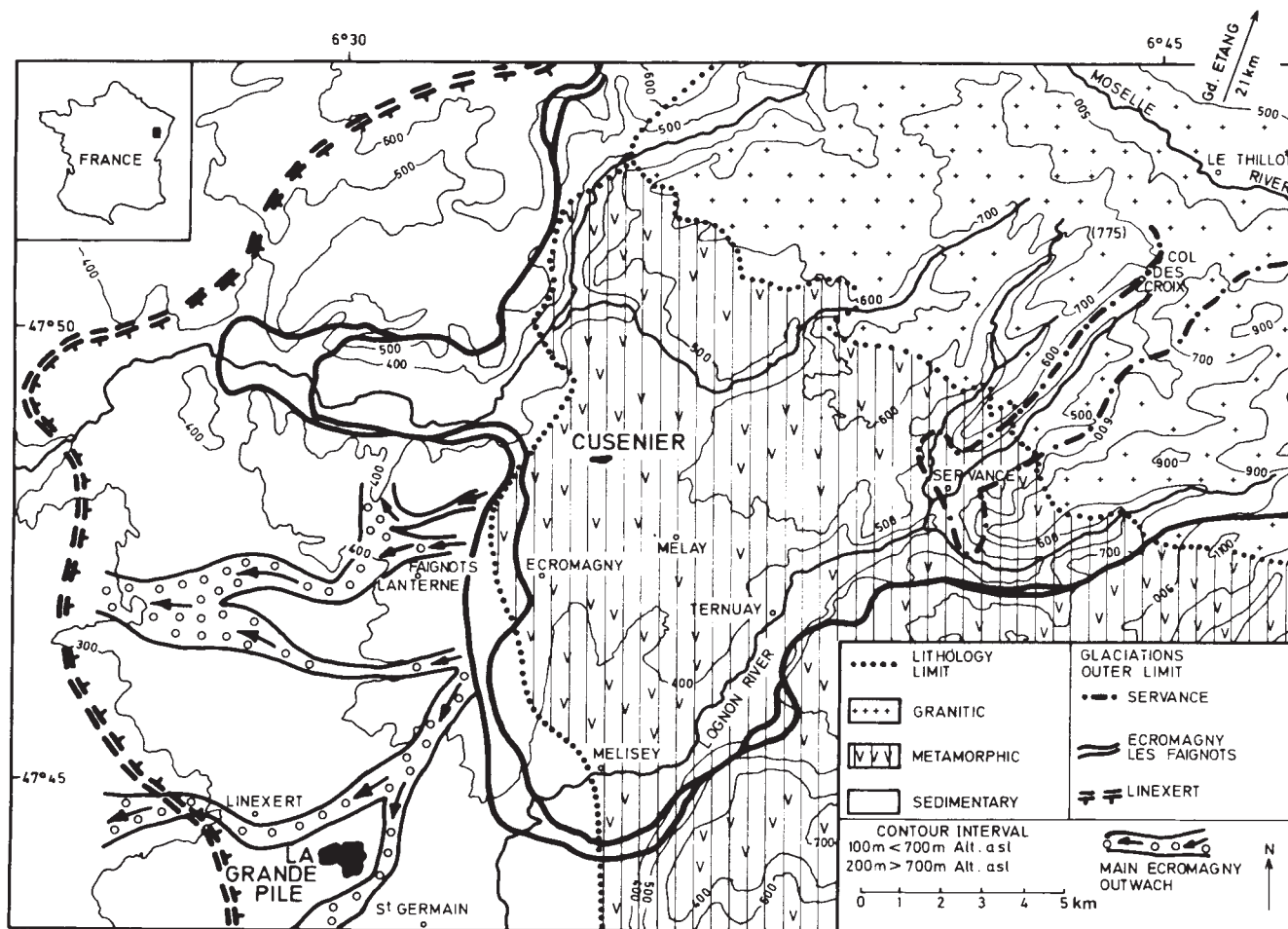


FIG. 1 Glaciations on the Ecromagny plateau.

indicative of sources on the Ecomagny Plateau. Between the St-Germain II and Pile interstadial, (~70–50 kyr ago), the source of the aeolian dust is the granites and sedimentary rocks. A sharp increase in the metamorphic content appears after the Pile interstadial at ~45–50 kyr, which we interpret as Ecomagny glacier outwash in the vicinity of the Grande Pile peat bog. During the Grand Bois interstadial at ~30 kyr there was a second shift of the mineralogical composition of aeolian dust back to granitic, indicating the absence of an ice sheet on the plateau.

The Cusenier peat bog is situated in a depression left by the retreating Ecomagny glacier. Like Grande Pile, it is isolated from any sources of detrital minerals other than local slope wash and aeolian transport. The core obtained in Cusenier bog in 1987 (Fig. 2) revealed peat, organic mud, lacustrine clays and silts overlying the lodgement and waterlain tills of the Ecomagny glacial advance⁷. The pollen diagram corresponds closely to the upper part of the Grande Pile core. It covers the end of the Grand Bois interstadial, the peak glacial tundra correlating with oxygen isotope stage 2 and the Holocene. The Melay ash is at 6.25 m depth and the Laacher See ash at 4.70 m. The Cusenier core shows that the glacier advance over the Ecomagny Plateau is older than the Melay ash and therefore older than 20,000 years. The mineralogical record of the Grande Pile core indicates that the outwash from the Ecomagny glacier became abundant at about the Pile interstadial, which suggests that the glacier advanced to its outermost moraines and retreated during the interval between the Pile and the Grand Bois interstadials (50–30 kyr).

The glaciers of the Servance stage did not build up high enough to cross the crest of the Ecomagny Plateau and only the upper part of Ognon valley was invaded by the ice, through the Col des Croix pass at 683 m a.s.l. (Fig. 1). The Grand Etang depression located inside the Servance moraine group, but outside the area shown in Fig. 1, has a till at the bottom followed by proglacial laminated clays, organic mud and finally peat. Only the Laacher See ash is present and the pollen diagram correlates with the uppermost part of the Last Glacial Maximum zone, the Late Glacial and the Holocene (Fig. 2).

Figure 2 also shows the smectite content of the clay mineral fraction. The smectite occurs in the Grande Pile mainly in the upper part of the Linxert sediments, in Mélisey I, in the Ognon complex and in the sediments between Grand-Bois and the Late Glacial. This last smectite event is also present in the Cusenier sediment. The smectites are well crystallized in the upper parts of both Linxert and the Last Glacial Maximum deposits, and in the Grand Etang sediments from the upper part of the Servance glaciation. The transition to the Late Glacial deposits is marked by a progressive decrease and finally complete disappearance of smectites and, in Grand Etang, by a progressive decrease of crystallinity.

The work of Blaise⁸ shows that there is close correlation between well crystallized smectite and a dry cold climate. It has even been suggested that not only is the preservation of smectite favoured by the cold climate but that it can also be formed under such conditions^{8,9}. We therefore associate the units containing smectite with periods of increased dryness. Because they appear to postdate the maximum expansion of the ice sheets in this area, the disappearance of the ice was probably due not only to warmer conditions but also to a decrease in precipitation. On the other hand the cold zones with absent smectite correspond to ice advances, which according to the palynological data were not the coldest ones in the core⁴. It therefore seems that the ice sheet grew in response to a large snow supply in a humid but not extremely cold climate.

Our evidence shows that a large body of piedmont ice existed in the southern Vosges at least once after the Lure interglacial and before the Last Glacial Maximum. The lower age limit is set by the St-Germain I temperate phase, the vegetation of which is incompatible with the presence of ice in the Vosges. The upper age, between 20 and 30 kyr, is recorded in the Cusenier core, which indicates that the ice sheet melted before the Melay ash and probably during the Grand Bois interstadial. The onset of the ice build-up is more difficult to fix—only when the ice sheet reached the Ecomagny Plateau could the fine detritus of metamorphic rocks registered in the Grande Pile cores since ~50 kyr be released into the local streams.

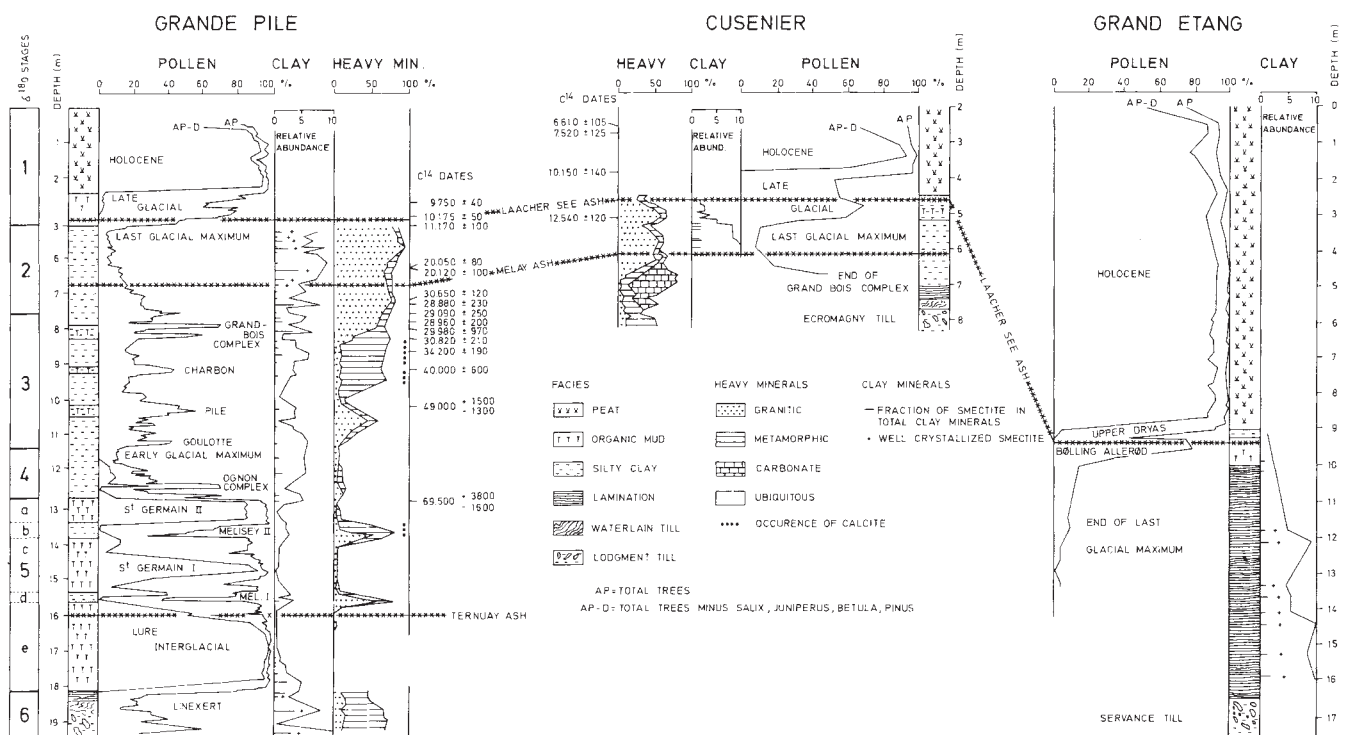


FIG. 2 Data from composite cores taken from bogs at Grande Pile, Cusenier and Grand Etang.

In our opinion two models are possible. According to the first, the ice build-up on the plateau started after St-Germain II, during the Early Glacial Maximum in oxygen isotope stage 4, and retreated during the Goulotte warming, at the start of oxygen isotope stage 3 some 60 kyr ago. During its retreat it left behind bare surfaces covered by tills rich in detritus of metamorphic rocks and these became the sources of the aeolian silt that reached the Grande Pile bog. According to this model the ice sheet present during oxygen isotope stage 4 would not have supplied any outwash and metamorphic detritus to the Grande Pile, which seems rather unlikely.

In the second model, which we favour, no ice was present on the Ecomagny Plateau during oxygen isotope stage 4—only some time after 50 kyr would the ice overflow the crest of the Ecomagny Plateau and, in two pulses, leave behind the twin Ecomagny moraines. This model bears some resemblance to the ice advances of Ontario lobe where tills of the Cherry Tree stadial separate the 48-kyr-old Port Talbot interstadial from the 23 to 32-kyr-old Plum Point interstadial¹⁰.

The degree to which the mineral composition of Mélisey I and Mélisey II silts indicate regional ice advances remains unclear. Whereas Mélisey I and the lower part of Mélisey II show a granitic source, the upper part of Mélisey II contains heavy minerals of metamorphic origin as well as detritic limestone. It is possible that the ice had reached the Ecomagny Plateau crest in Mélisey II (correlated with oxygen isotope stage 5b) because 80% of the metamorphic detritus is too coarse to be moved very far by wind. In addition, the absence of smectite suggests a large humidity supply, compatible with the growth phase of the glacier.

Whatever the details, a large ice advance must have reached the area between 50 and 30 kyr, because the sedimentation of Cusenier is well dated (Fig. 2), with continuous evolution from till to a complete and progressive glacier retreat sequence. The ice volume and extent were considerably larger than that during the Last Glacial Maximum.

The presence of a large ice mass at relatively low latitudes and elevations in western Europe long before the Last Glacial Maximum at 20 kyr favours models that link the mechanism of global cooling to changes in the snow supply and to the major reorganization of oceanic and atmospheric circulation in the North Atlantic^{11–13}. The position of the polar front in the North Atlantic fluctuated around 45° N during the Late Pleistocene^{14,15} and the largest range of surface air temperature also occurred in this belt^{16,17}. The front of the southern Vosges is close to this latitude, at ~48° N. The sequence of events in the sediments points to large variations in the moisture supply. It seems that the coldest phases were dry, resulting in a smaller glacier, and that growth of the ice sheet was accompanied by a large snow supply in a somewhat warmer climate. Our results also indicate that the glacial maximum may not have occurred at the same time around the world. □

U–Th ages obtained by mass spectrometry in corals from Barbados: sea level during the past 130,000 years

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THE study of the sea level record during the last glacial cycle has primarily proceeded indirectly by means of oxygen isotope measurements on foraminifera from deep-sea sediments^{1,2}. The direct approach of dating sea level indicators stagnated during the past decade, mainly because the samples required to complete our knowledge of the past glaciations are below the present-day sea level^{3–7}. Using the ¹⁴C ages of *Acropora palmata* samples collected by drilling offshore the island of Barbados, Fairbanks⁸ presented the first detailed chronology for the last deglaciation. This radiocarbon chronology is limited to the past 30 kyr because of the short half-life of ¹⁴C (5,730 yr); we must therefore rely on other dating methods to obtain information for the whole last glacial cycle. During the past four years ²³⁰Th–²³⁴U dating of corals by thermal-ionization mass spectrometry has been shown to be significantly more precise and accurate than the classical α -counting method^{9–14}. We have used this technique to measure U–Th ages in coral samples from the Barbados collection and from sub-aerially exposed outcrops (see also ref. 15). Here we present results bearing on the sea level record for the past 130,000 years; we conclude that the last deglaciation started 3,000 years earlier than previously thought and confirm that there were two surges in melt water⁸ at about 11 kyr and 14 kyr BP (before present).

The technique used for U–Th measurements is described elsewhere¹⁶. The activities and dates are listed in ref. 15 and Table 1. The agreement of our U–Th ages with the calibrated ¹⁴C ages is excellent for the past 10 kyr and we have shown that the two geochronometers can be reconciled between 10 and 30 kyr if we take into account the variation of the atmospheric ¹⁴C/C ratio inferred from the changes of the strength of Earth's magnetic dipole¹⁵.

We measured the sample RGF1-17-4 in duplicate at the accelerator mass spectrometry facility of Gif-sur-Yvette and determined that it was older than 48 kyr, the ¹⁴C dating limit¹⁶. This is in agreement with its U–Th age determined by mass spectrometry, 70.8 ± 0.6 kyr (Table 1).

The initial ²³⁴U/²³⁸U ratios are calculated from the measured ²³⁴U/²³⁸U, using the ages obtained from the ²³⁰Th–²³⁴U chronometer¹⁰. All corals younger than 70 kyr fall in the range of the modern sea water (1.14–1.15, ref. 9). This confirms that the Barbados corals grew in open sea water and that the U–Th system has not been strongly altered after deposition. By contrast, all corals older than 70 kyr have initial ratios between 1.15 and 1.165 (some samples have ratios as high as 1.2), in good agreement with the mass spectrometric results of Edwards¹⁰ and Chen and Wasserburg¹⁷ and with the recent α -counting results by Ku *et al.*¹⁸. The interpretation of ²³⁴U/²³⁸U ratios in the older corals from Barbados and other Caribbean islands will be discussed in detail elsewhere (B.H., E.B., R.G.F. and A. Zindler, manuscript in preparation). One possible explanation is that all corals have been contaminated by exogenous uranium early in their evolution and that all ²³⁰Th ages are essentially correct. An alternative explanation is that the ²³⁴U/²³⁸U ratio of sea

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TABLE 1 U-Th isotope data

Sample	Depth* (m)	^{238}U (p.p.m.)	$[\text{}^{230}\text{Th}]/[\text{}^{234}\text{U}]^\dagger$	$^{234}\text{U}/^{238}\text{U}$	$(^{234}\text{U}/^{238}\text{U})_{\text{init}}^\ddagger$	Th age (yr BP)
RGF12-30-2 #1	-78	3.22 ± 0.02	0.2458 ± 0.0016	1.1255 ± 0.0067	1.1368 ± 0.0074	$30,470 \pm 240$
RGF12-30-2 #2		3.46 ± 0.02	0.2428 ± 0.0014	1.1314 ± 0.0062	1.1431 ± 0.0068	$30,040 \pm 210$
RGF1-17-4	-57	3.62 ± 0.02	0.4840 ± 0.0027	1.1294 ± 0.0060	1.1582 ± 0.0076	$70,820 \pm 600$
RGF8-27-1 #1	-46	3.56 ± 0.02	0.5254 ± 0.0030	1.1517 ± 0.0063	1.1900 ± 0.0083	$79,400 \pm 700$
RGF8-27-1 #2		3.58 ± 0.01	0.5180 ± 0.0032	1.1501 ± 0.0061	1.1871 ± 0.0080	$77,800 \pm 800$
FS-13	18	3.34 ± 0.02	0.5634 ± 0.0031	1.1293 ± 0.0065	1.1661 ± 0.0087	$88,200 \pm 800$
AFZ-2	6	3.07 ± 0.01	0.6113 ± 0.0038	1.1145 ± 0.0064	1.1522 ± 0.0090	$100,500 \pm 1,100$
AFM3 #1	55	3.04 ± 0.02	0.6944 ± 0.0046	1.1077 ± 0.0072	1.1536 ± 0.0110	$125,100 \pm 1,700$
AFM3 #2	55	3.12 ± 0.01	0.6941 ± 0.0026	1.1065 ± 0.0037	1.1518 ± 0.0057	$125,100 \pm 1,000$

U-Th results obtained on *A. palmata* samples from Barbados (see ref. 15 for samples younger than 30 kyr). Duplicates were measured on different pieces of the sample. All errors are quoted at the 2σ level. All samples contain negligible amounts of calcite (X-ray diffraction measurement).

* Depth or altitude of recovery relative to present sea level.

† Measured atomic ratios were converted to activity rates using U-Th half-lives given in ref. 10.

‡ Initial $^{234}\text{U}/^{238}\text{U}$ ratios were obtained using Th ages to correct measured isotopic ratios.

water changed by $\sim 1.5\%$ since 100,000 yr BP. Therefore, all the corals with initial ratios between 1.15 and 1.165 have not been strongly altered and their ^{230}Th ages probably represent true ages of formation. By contrast, the corals with initial $^{234}\text{U}/^{238}\text{U}$ ratio higher than 1.17 have been contaminated by exogenous uranium and their ^{230}Th ages represent lower bounds on their ages of formation.

Figure 1 presents our sea level estimates obtained by U-Th dating, the normalized $\delta^{18}\text{O}$ curves determined by Shackleton¹⁹ and Labeyrie *et al.*²⁰ and the 65°N summer insolation curve²¹. In both cases the $\delta^{18}\text{O}$ chronologies have been orbitally tuned^{19,20}.

We have measured U-Th ages of coral samples corresponding to the last interglacial period from different Caribbean islands. These corals are not at the same altitude on the different islands because of the tectonic uplift that affects the Caribbean subduction zone. On the island of Curacao, which is thought to be relatively stable, corals from the last interglacial period are found at ~ 5 – 10 m above the present sea level²² in accordance with U-Th ages measured on corals collected in stable areas²³. By contrast, on Haiti²⁴ and Barbados^{25–27} those corals are found at altitudes between 40 and 60 m above the present sea level (on Barbados the uplift rates are even different in the southern and eastern parts of the island²⁷). By assuming that the sea level of the last interglacial period was 7 m above the present level, we have recalculated all the results obtained on Barbados corals using a linear tectonic uplift correction²⁷. The potential error on the sea-level reconstruction presented in Fig. 1 is smaller for younger ages and for the last deglaciation the uplift correction is practically negligible, of the order of the inherent error due to the habitat range of *A. palmata*.

Our high-precision results for the last interglacial period¹⁵ show that all the corals from this sea-level highstand are restric-

ted to a short period between 120 and 130 kyr in agreement with previous data^{11,12}. They are in disagreement with the conclusion by Winograd *et al.*²⁸ that the last interglacial period took place at ~ 140 – 150 kyr, which contradicts the Milankovitch theory (B.H., E.B., R.G.F. and A. Zindler, manuscript in preparation).

We now compare our U-Th age estimates obtained for coral samples corresponding to the last glacial period. For periods beyond the ^{14}C timescale (>30 kyr BP) there were previously only mass spectrometric results for two samples¹¹. Here we present five new samples which correspond to the interstadial periods of isotope stage 5, isotope stage 4 (sea-level low stand) and stage 3.

For the Barbados II terrace (Ventnor terrace in the Matthews²⁷ morphostratigraphic classification; isotope stage 5c) we measured an age of ~ 101 kyr (AFZ2) in agreement with the $\delta^{18}\text{O}$ chronologies and the mean age measured by α -counting (~ 105 kyr, refs. 25, 26; sample AFZ1 was dated by α -counting at 104 ± 8 kyr, ref. 26).

This age differs significantly from the results obtained by Edwards¹⁰ on sample FT-50 (112.0 ± 1.0 , 111.8 ± 1.3 , 112.3 ± 1.1 kyr) which would put this sea-level highstand at the time of an insolation minimum²¹. This sample was dated by α -counting¹⁸ at 103.6 ± 4 kyr, in disagreement with the results by Edwards¹⁰. This may be because sample FT50 is heterogenous because of alteration. Indeed, both thermal-ionization mass spectrometry and α -counting results^{10,18} show that this sample has an anomalously high initial ratio $^{234}\text{U}/^{238}\text{U}$ of ~ 1.17 – 1.18 .

Sample FS13 corresponds to Barbados I terrace (Worthing terrace; ref. 27; isotope stage 5a) and has been dated at ~ 88 kyr in excellent agreement with sample OC-51 dated by Edwards (87.5 ± 0.6 , 87.9 ± 0.7 kyr, ref. 10; sample FS3 was also dated by α -counting at 84 ± 8 kyr; ref. 26). These ages are, however, slightly older than the mean of the dates measured by α -counting

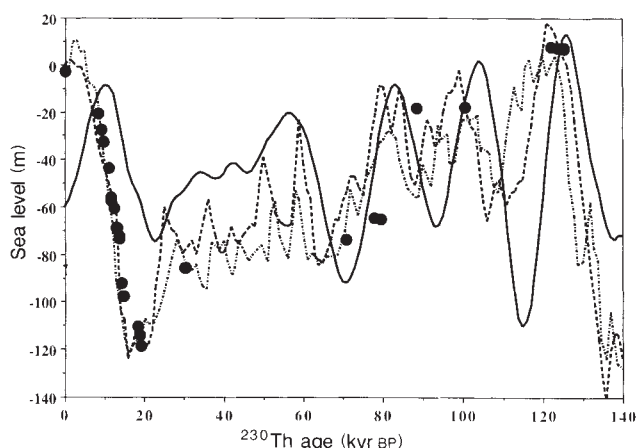


FIG. 1 *A. palmata* palaeodepth plotted against Th-U ages measured by mass spectrometry. To reconstruct this sea-level curve we applied linear corrections for uplift by normalizing the last interglacial high stands to 7 metres above the present sea level (see text). Barbados data are listed in Table 1 and ref. 15, data for samples C1 and C4 from Haiti are given in ref. 15. Dashed-dotted line, normalized $\delta^{18}\text{O}$ curve determined by Shackleton²⁰; dashed line, normalized $\delta^{18}\text{O}$ curve determined by Labeyrie *et al.*¹⁹; solid line: 65°N summer insolation²¹. ($\delta^{18}\text{O}(\text{‰}) = [(^{18}\text{O}/^{16}\text{O})_{\text{sample}} / (^{18}\text{O}/^{16}\text{O})_{\text{standard}}] - 1$, where standard is PDB.)

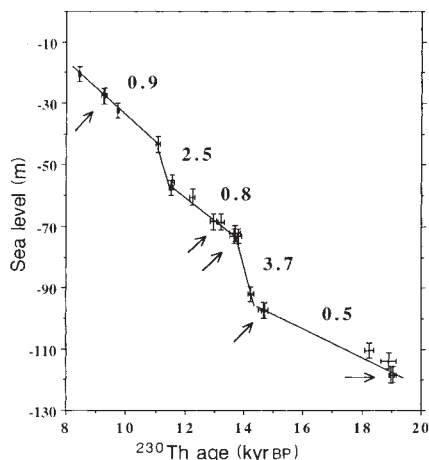


FIG. 2 Sea level during the last deglaciation. All samples are *Acropora palmata*. The U-Th age errors are quoted at the 2σ level. The samples marked by arrows correspond to duplicate analyses of the same coral sample by U-Th mass spectrometry. All duplicates are in agreement within error. A constant error of ± 2.5 m has been assumed for the sea level. The numbers correspond to the melting rates (metres per century) calculated from slopes of the linear regressions.

on Barbados I (closer to 82 kyr, refs 18, 25, 26), which was used in the $\delta^{18}\text{O}$ tuning (Fig. 1). The mass spectrometric U-Th data suggest an earlier age for isotope stage 5a and therefore a smaller time lag between the climate response and the orbital forcing.

Samples RGF1-17-4 and RGF8-27-1 were collected below discontinuities characterized by erosion and dissolution features typical of a prolonged sub-aerial exposure⁸. These samples were dated at 70 kyr and 79 kyr and can be considered as contemporaneous with the early part of isotope stage 4 in rough agreement with the $\delta^{18}\text{O}$ chronologies.

Sample RGF12-30-2 was also collected below a sub-aerial exposure. This critical sample indicate a sea level of -85 m at 30 kyr and gives a new independent constraint on the sea level during isotope stage 3. Our data differ significantly from the only other available estimate, based on classical U-Th and ^{14}C dating of a coral sample from the Huon Peninsula in New Guinea (-40 m at ~ 30 kyr, ref. 29). The result obtained in Barbados and New Guinea could be reconciled by a rapid decrease in the sea level of ~ 40 m in < 6 kyr at ~ 35 kyr (the 2σ error of the previous U-Th estimate is 5 kyr). This is supported by the reconstructions based on benthic $\delta^{18}\text{O}$ curve (Fig. 1) which exhibit high-frequency structures during isotope stage 3.

Figure 2 focuses on the coral record of the last deglaciation dated by U-Th. It can be seen that this abrupt climate event started before 19–18 Th kyr and was characterized by two large jumps dated at ~ 13.5 kyr and 11 kyr, called melt water pulses 1A and 1B⁸ by Fairbanks. The first rise corresponds to a dramatic rate of sea level change of ~ 3.7 metres per century, followed by a decrease down to < 1 metre per century and then by a second abrupt change of ~ 2.5 metres per century (Fig. 2). Those rates were already quantified by Fairbanks⁸ using the ^{14}C chronology. The better precision and accuracy given by the U-Th chronology confirm that the rates of the meltwater pulses were higher than 10^6 km^3 of continental ice per century.

These new results have several important implications for palaeoclimate studies: (1) The fact that the two abrupt sea level changes exist in both ^{14}C and U-Th timescales demonstrates that the two jumps in the sea level record⁸ and benthic $\delta^{18}\text{O}$ curves³⁰ are not artefacts of the ^{14}C timescale. (2) These results also suggest that the time lag between orbital forcing and ice-sheet response was overestimated when it was solely based on the ^{14}C chronology. The maximum value is of the order of a few kiloyears. Our results will help in choosing the climate time lag to refine the SPECMAP timescale³¹ and in calculating the conversion factor between $\delta^{18}\text{O}$ and sea level changes⁶. (3) Large discrepancies exist between data on the local sea level obtained by the ^{14}C methods and those determined using geophysical models that calculate the response of a layered viscoelastic Earth to surface load forcing^{32,33}. To explain the ^{14}C -dated sea level estimates and the recent tide-gauge data, very large Barents Sea ice sheet³² and/or significant melting of Antarctica³³ has been invoked. Our data demonstrate that the

last deglaciation started 3,000 years earlier than previously thought and this helps to explain the disagreement between observations and models (W. R. Peltier, manuscript in preparation).

Our mass spectrometric U-Th data provide a high-precision absolute chronology of the last glacial cycle. The Barbados sea-level curve has been augmented using data from submerged samples, enabling a close comparison with other chronologies inferred for the last glacial cycle^{18,19,31,34–37}. In particular, the sea level was below -70 m during isotope stage 4 and below -80 m for the end of stage 3. The deepest *A. palmata* sample is dated at ~ 19 kyr and corresponds to a sea level of -118 m for the Last Glacial Maximum. As demonstrated by Fairbanks⁸, the last deglaciation is characterized by two steps which are now dated by the U-Th method at ~ 11 kyr and 14 kyr. These two steps are the likely consequences of two dramatic meltwater surges which exceeded the rate of 10^6 km^3 of continental ice per century. □

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Three-liquid immiscibility and the origin of carbonatites

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THE relationship of rock types involved in carbonatite complexes has provoked much debate¹⁻⁴. One favoured theory of origin involves the immiscible separation of carbonate and silicate-rich liquids to produce the bimodal distribution of rock types typical of many complexes. Previous investigations of liquid immiscibility at crustal pressures in both natural^{5,6} and synthetic³ systems have demonstrated that immiscibility can produce much of the chemical diversity (in both major^{3,5,6} and trace elements⁷) of this group of rocks; however, arguments persist regarding the relative importance and genetic relationship of alkali-rich and alkali-poor carbonatites^{1,4,8-10}. Here we report the first results of new experiments at upper-mantle pressures, which show unexpected features indicating the existence of three immiscible liquids, one silicate- and two carbonate-rich, with surprising density relationships. These two carbonate liquids and the associated vapour could account for many carbonatite characteristics, while avoiding some problems encountered in deriving magmas of variable alkali content from a single carbonatite parent.

The photographs in Fig. 1 show selected areas of vertical sections through five different charges that have been rapidly quenched from 1,225 °C and 15 kbar. The majority of the experi-

ments are in the system $\text{SiO}_2\text{--Al}_2\text{O}_3\text{--CaO--Na}_2\text{O--CO}_2$ although minor constituents have been added in some runs. The composition of starting mixtures and the resulting phases are listed in Table 1 and plotted in Fig. 2. The charges generally consist of a silicate bead (diameter equal to the capsule width, ~5 mm) situated towards the base of the charge, with a mixed carbonate above and a single carbonate below. Vesicles are always present, demonstrating the existence of a vapour phase. With certain bulk compositions a few silicate phenocrysts may also be present.

We interpret these charges to represent three immiscible liquids for the following reasons. All three phases can be seen in spherical or droplet form displaying a range of sizes, in some cases coalescing to form larger areas. These spheres are at least one or two orders of magnitude larger than the maximum sphere size (2 µm) produced by metastable quench immiscibility¹¹ and boundaries are sharp (<1 µm). The composition of each phase seems to have been homogeneous before the quench and entrained vesicles support a liquid interpretation. Very similar phase compositions are produced from a wide range of starting mixtures so long as the bulk compositions plot within the three-liquid field of Fig. 2. This consistency is independent of the run duration or the textural relationship of the phases.

The presence of an almost pure Ca carbonate liquid at 1,225 °C requires further attention as the melting point of calcite at 15 kbar should be >1,500 °C (ref. 12). The Ca carbonate in these charges displays the morphology of a liquid, following the complex shape of the capsule wall and exhibiting a curved meniscus against other liquid phases. Dumbbell-shaped Ca carbonate inclusions have also been observed in the silicate liquid. Ca carbonate liquids do not quench to glass, even at the rapid

FIG. 1 *a-e*, Back-scattered electron (BSE) images taken on Cameca microprobe. *f*, Reflected-light photomicrograph. All images are orientated with the top of the charge uppermost; *a* and *e* are different areas of the same charge. *a*, Three liquids together. The lightest area (L_c), with many triangular cleavage pits, is almost pure Ca carbonate. This area shares a smooth curved boundary with a silicate glass (L_s) which contains a large crystal (xst) and a sphere of mixed Na-Ca carbonate (L_{mc}). At higher magnification this sphere is seen to consist of an intergrown, two-carbonate quench texture typical of a (homogeneous) mixed-carbonate liquid³. *b*, Lightest area on the left is the platinum capsule wall (Pt), this is in contact with an area of quenched Ca carbonate melt (L_c). The next area inwards shows the typical dendritic quench texture of the mixed Na-Ca carbonate (L_{mc}). The remaining area (L_s) is silicate glass. Vesicles (black) can be seen on the boundary between L_c and L_{mc} and in L_s towards the top of the photograph. *c*, Boundary between dendritic mixed carbonate (L_{mc}) and the lighter Ca carbonate (L_c) quenched liquids with spheres of silicate glass (L_s). *d*, Products of a run with BaO added to the starting composition (see Table 1, RB127). The medium grey, coalescing spheres (L_c) are Ca carbonate. The background lamellae (L_{mc}) represent another typical quench texture of a mixed Na-Ca carbonate liquid. *e*, Arrangement of the phases in the base of the charge. The darker area (L_s) is the bottom of the silicate bead and contains numerous phenocrysts. The area between the bead and the base of the capsule (bottom of photograph), is occupied by Ca carbonate liquid (L_c). The patches in L_c are clusters of small crystals of the same composition as the larger phenocrysts in the silicate (L_s) above. *f*, An area of Ca carbonate (L_c) in the base of a charge sharing a sharp curved meniscus with a mixed Na-Ca carbonate liquid (L_{mc}). The Ca carbonate contains a few small (50 µm) spheres of silicate glass which are difficult to see in reflected light. Note the curvilinear nature of the grain boundaries which form the mosaic pattern of the Ca carbonate area.

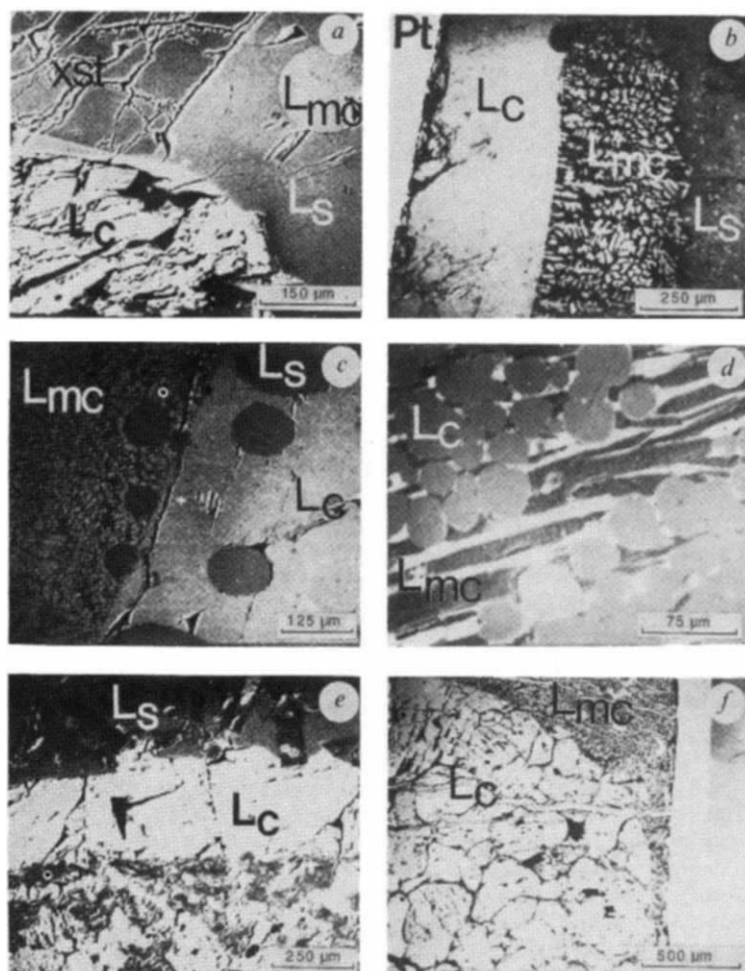


TABLE 1 Run data

Run	RB104	RB110	RB113	RB126	RB127	RB130*	RB132	RB133
Run time (h)	24	30	31	5	18	18	24	20
Bulk composition								
SiO ₂	4.20	20.61	22.33	20.61	20.88	22.33	21.61	20.93
Al ₂ O ₃	1.20	10.34	8.83	10.34	8.26	8.83	8.63	8.43
CaO	45.00	31.64	31.64	31.64	29.58	31.64	32.18	31.61
Na ₂ O	9.60	8.81	8.81	8.81	8.24	8.81	7.56	6.88
K ₂ O	—	—	—	—	—	—	1.36	—
BaO	—	—	—	—	5.05	—	—	—
MgO	—	—	—	—	—	—	—	2.39
CO ₂	40.00	28.60	28.39	28.60	27.99	28.39	28.66	29.76
Silicate liquid (L _s)								
SiO ₂	48.31	50.44	49.20	48.00	46.88	50.17	48.20	38.58
Al ₂ O ₃	19.70	20.66	18.13	21.79	19.73	20.00	20.10	16.67
CaO	19.11	13.77	20.58	15.94	17.34	16.56	15.20	25.52
Na ₂ O	10.88	12.13	10.09	10.27	10.01	11.27	9.10	6.45
K ₂ O	—	—	—	—	—	—	2.10	—
BaO	—	—	—	—	3.68	—	—	—
MgO	—	—	—	—	—	—	—	2.61
SF†	2.00	3.00	2.00	4.00	2.36	2.00	5.30	10.16
Mixed carbonate liquid (L _{mc})								
SiO ₂	3.42	2.88	3.17	2.77	3.30	2.94	4.20	4.41
Al ₂ O ₃	0.30	0.24	0.31	0.35	0.36	0.24	0.60	0.59
CaO	46.06	44.28	39.42	45.00	42.81	45.72	46.90	45.25
Na ₂ O	11.28	12.60	9.10	10.68	10.50	11.10	8.70	8.20
K ₂ O	—	—	—	—	—	—	1.00	—
BaO	—	—	—	—	6.41	—	—	—
MgO	—	—	—	—	—	—	—	1.92
SF	38.94	40.00	48.00	41.20	36.62	40.00	38.60	39.60
Ca-carbonate liquid (L _c)								
SiO ₂	0.28	0.20	0.30	0.40	0.41	0.25	0.28	0.89
Al ₂ O ₃	—	—	—	—	—	—	—	0.14
CaO	55.44	56.80	55.50	54.70	50.27	56.00	56.00	54.28
Na ₂ O	0.28	—	0.20	0.30	1.24	0.30	0.50	0.37
K ₂ O	—	—	—	—	—	—	—	—
BaO	—	—	—	—	4.21	—	—	—
MgO	—	—	—	—	—	—	—	0.39
SF	44.00	43.00	44.00	44.60	43.87	43.45	43.22	44.00

All runs at 1,225 °C and 15 kbar. Compositions are in weight per cent.

* The starting mix of RB130 included 20 wt% of calcite cleavage rhombs.

† These analyses were obtained using an electron microprobe, which cannot analyse volatile components. SF indicates the shortfall for the phase and in the case of the carbonates this is equivalent to the expected stoichiometric amount of CO₂. The volatile component producing the shortfall in the silicate phase has been analysed using infrared and preliminary result show that it is composed mainly of CO₂ but some water is also present.

quench rate of these experiments (1,225 °C to 700 °C in 8 s), and the outline is filled by either a single crystal or a mosaic of crystals each with optical continuity and sets of parallel cleavage (see Fig. 1f). The small individual globules of Ca carbonate typical of runs in the low-alkali two-liquid field (see Fig. 3b, c) are also shown to be crystalline by X-ray diffractometry.

To try to demonstrate that Ca carbonate is molten under these conditions, calcite cleavage rhombs were arranged throughout a capsule, replacing most of the usual CaCO₃ powder in the starting mix. These rhombs were not present in the resulting charge, which had a single area of Ca carbonate in the base and the usual phase compositions (see Table 1). The low liquidus temperature of the Ca carbonate may be due to the presence of water in the charges. Although every attempt was made to ensure that the starting materials were dry, infrared analysis of the silicate glass bead indicated the presence of water, possibly formed as a result of hydrogen diffusion through the platinum capsule wall during the run¹³.

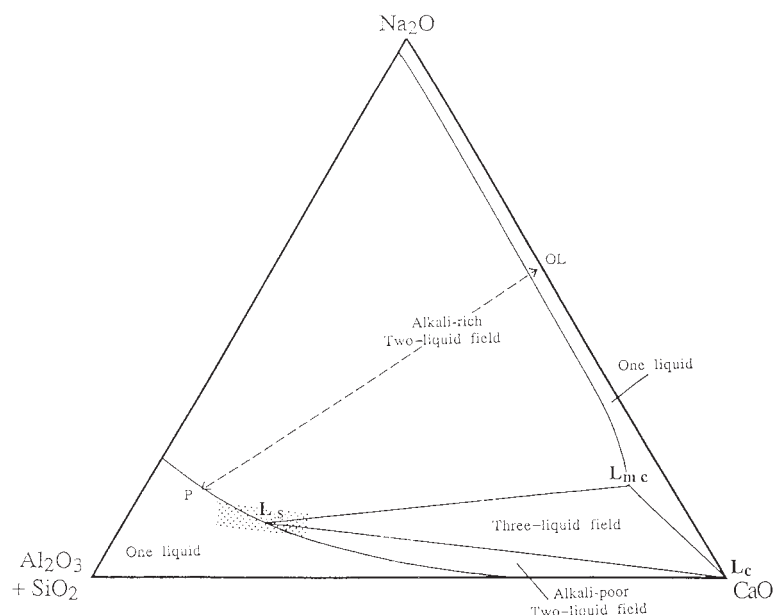
Another unexpected feature of the Ca carbonate liquid is its position towards the base of the capsule. Although there have been no direct measurements of Ca carbonate liquid density, an estimate of 2.2 g cm⁻³ has been made by comparison with Na carbonate¹⁴. These experiments suggest that at 15-kbar Ca

carbonate liquid is not only more dense than the Ca-Na-carbonate liquid, but also of similar or higher density to a conjugate nephelinite-type silicate liquid. The contrast in physical behaviour between alkali-rich and alkali-free carbonates, is further demonstrated (see Fig. 3) in the two-liquid fields, above and below the three-liquid stability field (see Fig. 2).

All runs show evidence of a vapour phase, but it has not been possible to obtain quantitative analyses. Mass balance considerations, however, indicate that a substantial amount of Na partitions into the vapour, and in similar experiments at lower pressure Koster van Groos and Wyllie¹⁵ identified a vapour containing "much" dissolved Na, which they suggested could represent an agent of fenitization.

The results of these synthetic experiments provide a new flexibility to carbonatite genesis modelling. The addition of further components to this simple system is important in evaluating the relevance of any model to natural rocks and we have included the results of additions of minor quantities of MgO, K₂O and BaO in Table 1 to give some indication of how some of these components may behave. A comparison of the results from natural⁵ and synthetic³ systems, however, shows the value of the simple analogy in interpreting the more complex situation.

FIG. 2 The compositions of the three liquids at 1,225 °C and 15 kbar are plotted on a pseudo-ternary wt% diagram. To enable the representation of five components, SiO_2 and Al_2O_3 are combined in one corner and a projection is made from CO_2 ; carbonate liquids contain 44–60 wt% CO_2 and silicate liquids 2–5 wt%. Any starting composition within the three-liquid triangle will produce L_s , L_{mc} and L_c in variable quantities. These compositions appear reasonably constant despite a variety of SiO_2 : Al_2O_3 ratios in the starting composition thereby justifying the combination of these 'network formers' in one corner. To enable natural rock compositions to be plotted on this diagram, K_2O is added to Na_2O , and FeO , Fe_2O_3 and MgO are added to CaO (after ref. 5). The composition of an Oldoinyo Lengai natrocarbonatite (OL) is shown along with the experimental conjugate silicate liquid which corresponds to a phonolite (P). The dotted area is the range of typical nephelinites (and ijolite).



Although many conclusions can be drawn from these results, we draw attention to the following possibilities.

Immiscibility could be induced as a CO_2 -saturated nephelinitic magma cooled to intersect the two- or three-liquid solvus (these experiments show one liquid at 1,325 °C and two at 1,250 °C), or by an increase in the CO_2 concentration of the liquid owing to fractionation of non-carbonate-bearing phases. LeBas² suggests that magma collects in a reservoir at the mantle/crust boundary, building up CO_2 by the precipitation of olivine and clinopyroxene. The parental magma would plot closer to L_s in Fig. 2 than the experimental starting compositions

and the proportion of both carbonate liquids would consequently be much smaller.

If three liquids were produced then the low-density carbonate liquid corresponding to L_{mc} would separate first, perhaps reaching the surface to produce carbonatite lavas or lapilli tuffs. With the exception of Oldoinyo Lengai, all natural extrusive carbonatites are calcitic^{4,16} (or dolomitic¹⁷), most containing calcite phenocrysts. Crystallization of these phenocrysts at atmospheric pressure from the observed melt composition is difficult to accept because of the problem of dissociation. One possible explanation is that the original lava had a substantially higher alkali content,

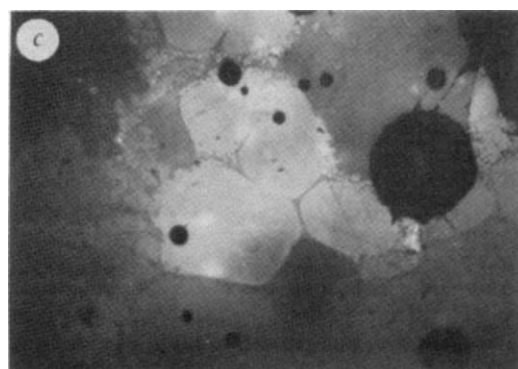
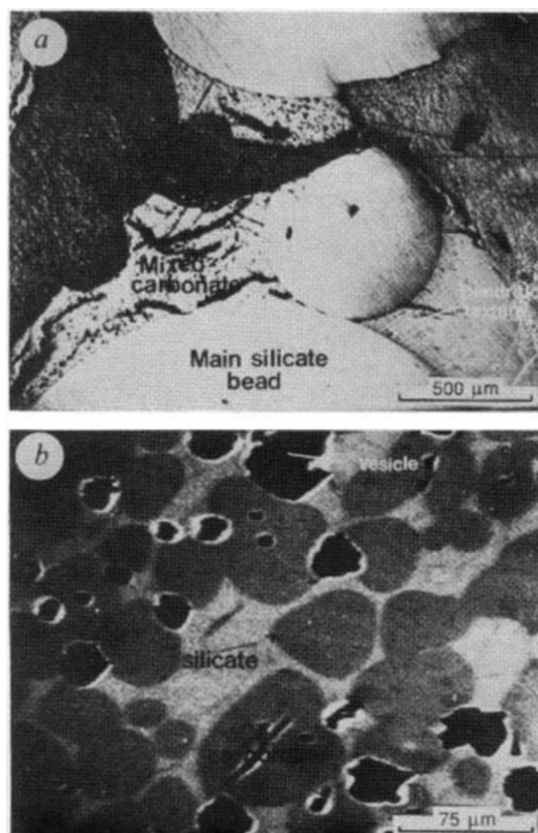


FIG. 3 *a*, Products of a run in the alkali-rich two-liquid field. This reflected-light photomicrograph shows horizontal sections through a main (and minor) silicate bead that is surrounded by alkali-rich carbonate quenched liquid with the typical dendritic texture, just visible at this magnification. The composition of the silicate glass is comparable to a phonolite and the carbonate is similar to the natrocarbonatite of Oldoinyo Lengai. *b*, Runs in the alkali-poor two-liquid field contain numerous alkali-free carbonate globules surrounded by a silicate glass. This BSE image shows globules of Ca-carbonate which never separate into a single area even if left for four days although this immiscible texture is achieved in less than a few hours. *c*, Reflected-light photomicrograph of the charge from a run just below the three-liquid field. Small globules of Ca-carbonate are seen coalescing to form larger globules (of similar size to those in *b*), which appear to collect in the base of the capsule and deform to give curvilinear boundaries. This run was quenched after only 5 h and suggests that a texture similar to that in Fig. 1f could be produced eventually.

which was subsequently modified by meteoric water. The discovery of what appeared to be calcite pseudomorphs after primary nyerereite, seemed to support this conclusion^{8,10}. This led LeBas⁸ to suggest that a natrocarbonatite such as Oldoinyo Lengai (75% alkali carbonate) would be the parent of most carbonatites, the high alkali content escaping as fenitizing fluids to leave compositions capable of crystallizing primary calcite and nyerereite. But many workers dispute the inferred existence of nyerereite¹⁶⁻¹⁸, and Gittins⁴ has shown that only 15 wt% Na₂CO₃ is required in a calcitic carbonatite to allow calcite to crystallize at atmospheric pressure as long as >5 wt% fluorine is also present. Such a lava would correspond to L_{mc} in our study, with the small amount of alkalis being easily lost from the ground mass. The role of fluorine is important in these rocks and although the low liquidus of the Ca carbonate (L_c) in these experiments is attributed to accidental H₂O, a small amount of fluorine would produce a similar effect⁴.

Models in which fenitizing fluids emanate from a natrocarbonatite, removing enough alkalis to produce the typical carbonatite composition, may be criticized on several points. As suggested above, the evidence for alkali-rich extrusive rocks has been seriously questioned^{9,16}, and there is no clear evidence from intrusive carbonatites for any intermediate compositions or for the large volume reductions and replacement features that would be produced by alteration of such rocks. Some compositionally zoned silicate minerals in intrusive carbonatites actually show an increase in alkalis from cores to rims¹. The occasional absence of fenites around some carbonatites and a closer spatial association with the silicate rocks in some complexes^{19,21} could be explained by a vapour phase associated with all the magmas rather than by a vapour emanating from one in particular (again, fluorine may be important).

The intrusion sequence with carbonatites cross-cutting earlier silicate rocks has led to criticism¹ of models involving immiscibility owing to the presumed low density of carbonate liquids. But the Ca carbonate seems to be the most dense of these liquids at 15 kbar and would therefore be intruded late in a complex, perhaps partially crystallized.

These preliminary experimental results show that three-liquid immiscible fractionation may be possible under upper-mantle conditions and indicate that a carbonate liquid may be more dense than a conjugate silicate liquid. Further investigation involving the addition of components such as MgO, FeO, TiO and in particular phosphorus and fluorine may help to evaluate further the relevance of this mechanism to carbonatite genesis. □

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Pteropod abduction as a chemical defence in a pelagic antarctic amphipod

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THIS study documents an example of an invertebrate that cannot defend itself chemically (an amphipod) increasing its chances of survival by capturing and carrying a species that can (a pteropod). Although chemical defences are found in a wide variety of marine invertebrates¹⁻⁴, few studies have established the extent to which these chemicals will deter predators⁵⁻⁷, and even fewer have investigated how one organism might exploit another's chemical defence to protect itself⁸⁻¹⁰. These chemicals are usually ingested or sequestered in the host's tissue^{8,9}. Several species indiscriminately decorate themselves with potentially toxic organisms or are passively fouled with chemically defended organisms^{11,12}. These seem to be commensalisms or sometimes mutualisms. Our example has benefits and costs for the carrier, but only costs to the captive. The antarctic marine food web not only has a variety of chemically defended organisms¹³⁻¹⁶, contrary to earlier predictions¹⁷⁻¹⁹, but has at least one unusual symbiosis that makes use of noxiousness.

The pelagic hyperiid amphipod *Hyperietta dilatata* is one of the most common prey of the abundant, plankton-eating fish *Pagothenia borchgrevinkii*^{20,21}. Moreover, this amphipod is found in the stomach contents of at least five other common antarctic fish (*P. bernacchi*, *P. hansonii*, *Trematomus centronotus*, *T. nicole*, *Gymnodraco acuticeps*)^{21,22}. *Hyperietta dilatata* and other members of the Hyperidae lack conspicuous morphological attributes such as spines, which might ward off fish predators²³. But in McMurdo Sound, Antarctica, during the austral summer, we observed amphipods selectively carrying small individuals of the abundant pelagic pteropod *Clione limacina* (Table 1; Figs

TABLE 1 Frequency of *Hyperietta dilatata* carrying *Clione limacina* at two sites in McMurdo Sound, Antarctica

	No. amphipods with pteropods	No. amphipods without pteropods	% Amphipods with pteropods
Coastal site (9 m depth)			
Total	225	80	74 (range 61-81)
Offshore site (50 m depth)			
Total	93	1,394	6 (range 5-10)
Offshore site (7-10 m depth)			
Total	17	35	33 (range 29-35)

Plankton nets (0.75-m) equipped with 505-μm mesh were deployed at depths of 9 and 50 m at two sites (shallow site near shore, 3 km northeast of Hut Point on Ross Island; deep site offshore, 1 km south of McMurdo Station). During November 8-27, 1989 the coastal site was sampled 4 times, whereas the offshore deep and shallow sites were sampled 6 and 2 times, respectively. Plankton nets were positioned horizontally and allowed to fish passively for 24 h. The numbers of paired and unpaired pteropods and amphipods were noted immediately. Ratios of amphipods to pteropods were roughly equivalent at both coastal and offshore sites (range, 1:1-1:3 amphipods to pteropods). Relative mean abundances of pteropods were 267 per 24 h of net deployment at the shallow site, and 32 and 653 per 24 h net deployment at the 7-10 m and 50 m offshore sites, respectively. Absolute abundances (number per 1,000 m³) of *C. limacina* and *H. dilatata* near McMurdo Station are reported by Foster *et al.*²⁰.

TABLE 2 Fish acceptance/rejection of the pteropod *C. limacina* and the amphipod *H. dilatata* when carrying pteropods

	<i>Clione</i>	Cod pieces	Agar with <i>Clione</i>	Agar without <i>Clione</i>	<i>Hyperietta</i> with <i>Clione</i>	<i>Hyperietta</i> without <i>Clione</i>
Fish accept	0	23	0	20	2*	13
Fish reject	23	0	20	0	10	0
Fisher exact test (<i>P</i>)	<0.001		<0.001		<0.0001	

Living *C. limacina* (2–20 mm length) were offered individually to a group ($n=97$) of *P. borchgrevinki* (14–20 cm length). Preliminary observations revealed that a prey item presented to the group of fish caused a general feeding response with many individuals (>50%) attempting to feed. Each pteropod was paired with a similarly sized piece of flesh from the antarctic cod *Dissostichus mawsoni*. In 23 independent feeding trials, all cases resulted in multiple violent rejections of the pteropod from the mouths of at least 10 of the fish. All fish tissues were immediately ingested. A similar experiment was performed using standardized agar blocks (0.5×0.5×0.5 cm) containing a 1% dry weight concentration of fish meal. Experimental blocks were prepared with 1% fish meal and 10% (wet weight) whole homogenized *C. limacina*. In each of 20 trials, at least 10 different fish rejected agar blocks containing a 1% fish meal and pteropod homogenates, whereas fish rapidly ingested control agar blocks containing only 1% fish meal. Living individuals of *H. dilatata* and those carrying *C. limacina* were attached with a small amount of silicon grease to the end of a fine needle on a glass rod. Individual amphipods or amphipods carrying pteropods were rubbed lightly against the mouths of individual *P. borchgrevinki* ($n=17$ fish; 14–20 cm length) until taken into the mouth. The order of feeding trials was randomized. All amphipods without pteropods were ingested ($n=13$), but those carrying pteropods were violently spat out (8 with pteropod attached and 2 with pteropod separated from amphipod). In two other instances (*), the pteropod was separated from the amphipod in the mouth and only the pteropod ejected, indicating how important it is that the amphipod should tightly grasp the pteropod.

1 and 2a). Amphipods were observed *in situ* through holes drilled in the sea ice and after capture by plankton nets. In both cases, we observed individuals carrying pteropods. In several experiments we demonstrated that *C. limacina* is chemically defended against predation by *P. borchgrevinki* (Table 2). Field studies have shown that although *C. limacina* is abundant, it is not eaten by *P. borchgrevinki*²⁰. The chemical nature of the distasteful compound is not known, but it may be either derived from the diet or synthesized *de novo* as in other shell-less marine gastropods²⁴.

In a laboratory experiment we investigated whether the pteropod-carrying behaviour of amphipods could provide a chemical defence against fish predation. Individual *H. dilatata* (controls) and *H. dilatata*–*C. limacina* pairs (amphipods were seen always to carry only one pteropod) were collected from 9 m below the sea ice and offered to individual *P. borchgrevinki* in a randomly determined sequence. We recorded the numbers of pteropod-paired and unpaired amphipods ingested or tasted and then rejected. Observations were also made on the condition of the rejected individuals.

Pagothenia borchgrevinki showed a significant ($P<0.0001$; Fisher exact test) rejection for *H. dilatata* carrying *C. limacina*.

In all cases, amphipods that were not carrying pteropods were readily eaten, whereas almost all of those carrying pteropods were rejected (Table 2). Moreover, some fish seemed visually and/or chemically to detect amphipods carrying pteropods, frequently approaching them and then swimming away. Avoidance may be related in part to the orange coloration of both the amphipod and the pteropod, providing fish with a strong visual

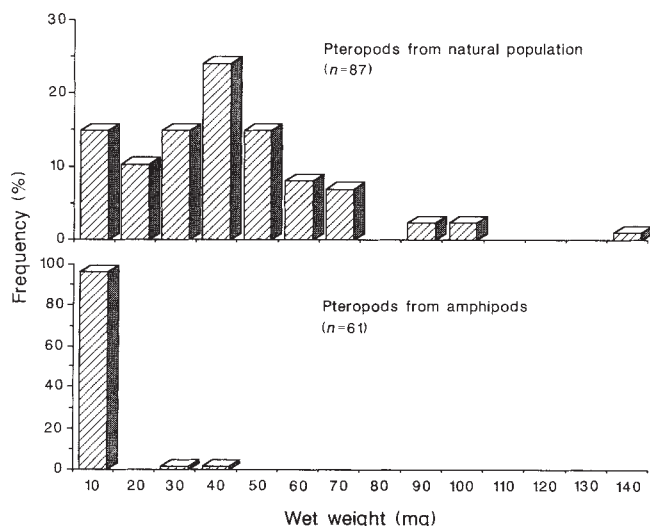
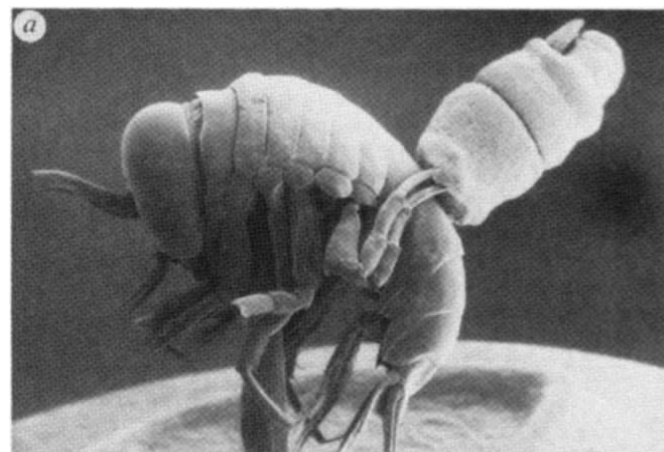


FIG. 1 Size frequency histogram showing the relative size (wet weights) of pteropods (*C. limacina*) sampled from the natural population, compared with individuals removed from the grasp of the amphipod *H. dilatata*. Pteropods removed from amphipods are significantly ($P<0.0001$; Mann Whitney U-test) smaller than those sampled from the natural population.

FIG. 2 a, Scanning electron micrograph showing the amphipod *H. dilatata* carrying the chemically defended pteropod *C. limacina*. Magnification is about 50×. b, Scanning electron micrograph showing the sixth and seventh pereopods of the amphipod *H. dilatata* grasping the pteropod *C. limacina*. Magnification is roughly 500×.

cue. Although fish violently (with head shakes) ejected amphipod-carrying pteropods from their mouths, amphipod-pteropod pairs were generally not separated upon ejection, and in no instance were the amphipods or pteropods killed. Moreover, coupled individuals did not separate when handled or placed into a 10% solution of $MgCl_2$, reflecting the great tenacity with which the sixth and seventh pereopods of *H. dilatata* grasp *C. limacina* (Fig. 2b). Amphipod-pteropod pairs observed in aquariums over a one-week period remained coupled. Uncoupled amphipods presented with swimming pteropods were observed to swim rapidly towards a pteropod and grasp the mantle posteriorly with the pereopods. One set of the sixth and seventh pereopods was then attached (Fig. 2b) and the retracted pteropod rotated dorsally. The opposing set of sixth and seventh pereopods was then attached, securing the pteropod against the abdominal segments for carrying (Fig. 2a).

This study demonstrates that the pelagic hyperiid amphipod *H. dilatata* captures and carries the pteropod *C. limacina* to defend itself chemically from fish predation. Abducted pteropods tightly retract and therefore do not feed, although some uptake of dissolved organic material may occur across the body wall. Nevertheless, we never found dead pteropods. Although other hyperiid amphipods, including *H. dilatata*, commonly associate with scyphozoans²⁵⁻²⁷, this is one of the few pteropod associations in this group. Trade-offs for the amphipod are involved in this symbiotic association. The benefits of pteropod carrying must be balanced against the increased energy expenditure necessary to counteract sinking when carrying a negatively buoyant mass. This is reflected in significantly reduced swimming speed. Swimming speeds (mean, $\pm$ s.d.) were 88.1 ± 22.1 ($n = 10$) and 53.5 ± 19.0 ($n = 10$) mm per second in individual and paired amphipods, respectively ($P < 0.01$; Student's *t*-test). Slower movements may impact both the ability of *H. dilatata* to feed and to avoid predators. Because abduction is facultative, amphipods may be able to adjust their defence to ambient predation risk. Whatever the cost-benefit relationship may be, the high incidence of pteropod carrying, particularly in shallow water where *P. borchgrevinki* is most abundant²⁰, indicates that this unusual behaviour is an important adaptation for a species that occurs together with a highly visual predator in the clear waters surrounding Antarctica. □

Arachidonic acid metabolites as mediators of somatostatin-induced increase of neuronal M-current

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THE M-current (I_M) is a time- and voltage-dependent K^+ current that persists at slightly depolarized membrane potentials¹. I_M is reduced by muscarinic cholinergic agonists and certain peptides, and is thought to be responsible in part for the slow and late slow excitatory postsynaptic potentials in sympathetic neurons¹⁻³. Recently, we reported⁴ that I_M in hippocampal neurons was also augmented by somatostatin-14 and -28 suggesting that two different receptors reciprocally regulate one neuronal channel type (ref. 4; see also ref. 5). Muscarinic effects on I_M may be mediated by various components of the phosphatidylinositol phosphate pathway^{6,7}. We now report the involvement of a different second messenger pathway, that generated by phospholipase A_2 ⁸⁻¹⁰, in the somatostatin-induced augmentation of I_M in hippocampal cells. This pathway generates arachidonic acid from which leukotrienes can be produced by lipoxygenases. We find that the I_M -augmenting effects of somatostatin are abolished by two substances that can inhibit phospholipase A_2 ¹¹, quinacrine and 4-bromophenacyl bromide, and that both arachidonic acid and leukotriene C_4 mimic the effects of somatostatin-14 on hippocampal pyramidal neurons *in vitro*. Arachidonic and somatostatin effects are blocked by a lipoxygenase inhibitor, implicating an arachidonic acid metabolite, perhaps a leukotriene, in the somatostatin effect.

We studied a total of 90 CA1 pyramidal neurons, with resting membrane potentials of -67 ± 0.5 mV and action potentials of 101 ± 1 mV (mean $\pm$ s.e.m.). A preliminary account of some of the data appears elsewhere¹². We tested somatostatin-14 (SS14) and elements of the phospholipase A_2 (PLA₂) system on the I_M , using voltage clamp at holding potentials of -38 to -50 mV (mean -44 mV). Somatostatin-14 ($1 \mu M$) increased the amplitude of the I_M relaxation by 50–55% in 33 of 46 cells (Figs 1 and 2a, e). This was associated with an outward shift of baseline (holding) current relative to control and with a conductance increase (Fig. 1a, and 2a, b). The remaining 13 cells did not show an I_M response to somatostatin and were not studied further.

Quinacrine ($8 \mu M$; eight of eight cells) and 4-bromophenacylbromide (4-BPB, $10 \mu M$; six of seven cells) abolished somatostatin-induced augmentations of I_M (Fig. 1a, b). Neither quinacrine (four cells) nor 4-BPB (four cells) alone in the superfusion medium had a measurable effect on I_M . Superfusion of arachidonic acid (120 – $150 \mu M$) augmented I_M relaxations by 50–75% in 28 of 39 cells (Figs 1c and 3a), increased input conductance and caused an outward shift of baseline current at both resting and holding potentials around -45 mV. The remaining 11 cells were not responsive to arachidonic acid and were not studied further.

The Q-current seen in hippocampal pyramidal neurons¹³ also produces an inward current relaxation with hyperpolarizing voltage steps, although only from hyperpolarized holding potentials (more negative than -70 mV). I_Q , but not I_M , can be inhibited by external Cs^+ (ref. 13), without an effect on the somatostatin-induced enhancement of I_M (ref. 4). As with somatostatin, arachidonic acid elicited I_M augmentation in three

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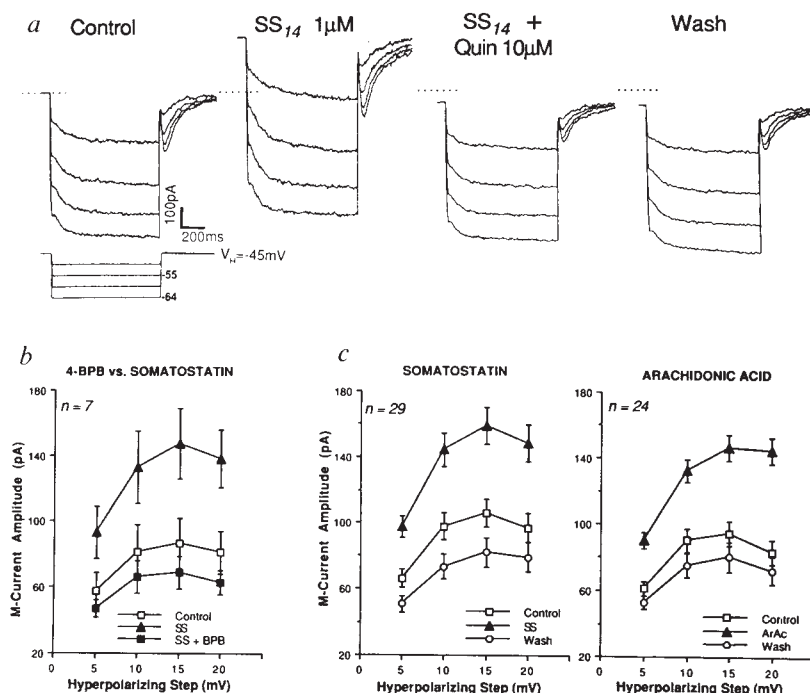
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FIG. 1 *a, b*, PLA₂ inhibitors block effects of SS14. *a*, Digitized records from a pyramidal neuron of superimposed inward current responses to the voltage protocol shown below the left current traces, from a -45 mV holding potential. With this protocol, deactivation of the outward I_M persisting at -45 mV appears as a slow inward current 'sag' (relaxation) following the instantaneous (ohmic) inward current drop. Somatostatin increases the I_M relaxation amplitude, associated with an outward baseline (steady state) current (dashed line = control baseline current). Somatostatin also increases overall conductance at this holding potential (compare steady state current amplitudes). The PLA₂ inhibitor quinacrine (quin; $10 \mu\text{M}$) significantly ($P < 0.01$) blocks all three effects of somatostatin. Time between each current panel in this and subsequent figures is 8–12 min. This and subsequent digitized ('raw') current figures (but not those used for curve fitting; see methods) were filtered digitally below 5 ms. *b*, 4-bromophenacyl bromide (4-BPB; $10 \mu\text{M}$), another PLA₂ inhibitor (perfused together with SS14) also significantly ($P < 0.01$) blocked the somatostatin-induced I_M increase (averaged $\pm$ s.e.m. from seven cells). *c*, Arachidonic acid (ArAc; right panel) mimics the effects of SS14 (left panel) on I_M relaxation amplitude: average data obtained only from 29 of 42 (SS) or 24 of 34 (ArAc) cells clearly responding to these substances. Both somatostatin (1 – $1.5 \mu\text{M}$) and arachidonic acid (120 – $150 \mu\text{M}$) significantly ($P < 0.01$) augmented the I_M relaxation amplitude to about 160% of control in these responding cells. In this and most subsequent analyses, I_M measures were averaged from all cells tested with a given drug at each command and submitted to a 2-factor (drug $\times$ voltage step) analysis of variance for repeated measures (each voltage step), with Newman-Keuls post-hoc tests (allowing estimation of P values between any two groups) used to test specific drug effects.

METHODS. Rat transverse hippocampal slices (350 – $400 \mu\text{m}$ thick) were completely submerged and continuously superfused with warm (30 – 31°C), gassed (95% O_2 , 5% CO_2) artificial cerebrospinal fluid (ACSF). The composition of the ACSF was (mM): NaCl, 130; KCl, 3.5; NaH_2PO_4 , 1.25; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5; CaCl_2 , 2.0; NaHCO_3 , 24; and glucose, 10. Intracellular and voltage clamp recording from CA1 pyramidal neurons was done with micropipettes filled with KCl (3M ; 50 – $80\text{M}\Omega$). Drugs and peptides were added to the ACSF (with tetrodotoxin, 0.5 – $1 \mu\text{M}$) in known concentrations. Arachidonic acid and leukotrienes were kept at -80°C and used immediately after suspension; the lipids were dried under nitrogen and dissolved in the ACSF by sonication. NDGA, 4-BPB, and leukotrienes were superfused with 0.1% dimethylsulphoxide (DMSO), and arachidonic acid with 0.25% DMSO. DMSO alone in the superfusion medium had no significant effect on seven cells (data not shown). Indomethacin was superfused with 16 mM ethanol, which alone did not affect I_M . We used the single electrode voltage-clamp recording mode (Axoclamp preamplifier), with switching frequencies of 3–4 kHz. Electrode

of three neurons bathed with 2mM Cs^+ (Fig. 2*a*). Neither somatostatin nor arachidonic acid significantly altered reversal potentials for the I_M relaxations (Fig. 2*a*). At holding potentials of -40 mV, both somatostatin and arachidonic acid increased M conductance from about 4–5 (control) to 8–9 nS (Fig. 2*b*). Although there was a trend for the I_M time constant to increase with superfusion of either somatostatin or arachidonic acid (cf ref. 5), this effect did not reach significance when averaged across all responding cells (data not shown).

Superfusion of indomethacin (8 – $10 \mu\text{M}$) to block cyclooxygenase action (an alternative branch of the arachidonic acid pathway, leading to prostanoids) did not significantly antagonize somatostatin-induced (eight cells) or arachidonic acid-induced (five cells; Fig. 2*c*) augmentation of I_M . Indeed the somatostatin (two cells) and arachidonic acid effects (two cells) were sometimes increased by indomethacin (Fig. 2*c*); indomethacin alone had no effect on I_M (three cells). Cyclooxygenase blockade might lead to shunting of arachidonic acid toward the lipoxygenase path¹⁴, thus accounting for the occasional enhancement of somatostatin effects seen with indomethacin. Prolonged indomethacin superfusion times (15 – 20 min), however, reduced somatostatin-induced increases in I_M to below control levels in four out of eight cells. Such late actions could reflect nonspecific inhibition of PLA₂ (ref. 15) as long superfusions with



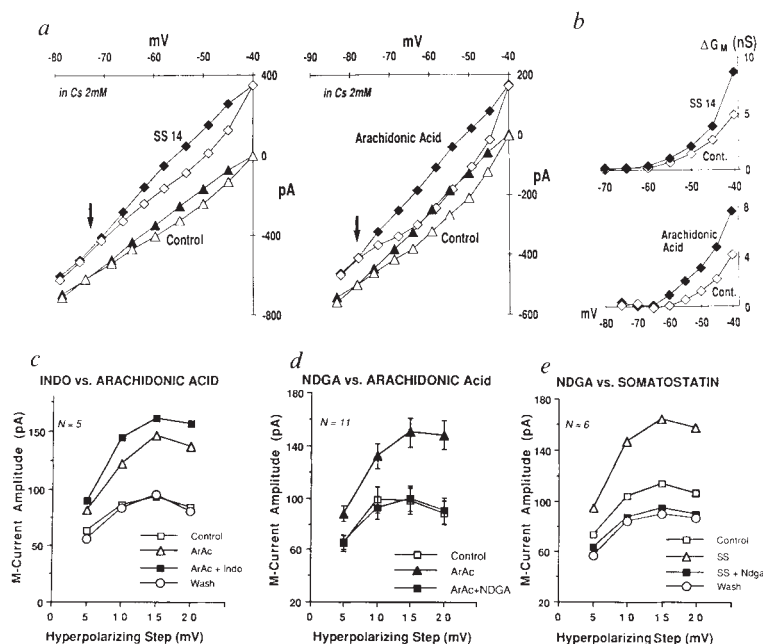
settling time and input capacitance neutralization at the headstage were monitored continuously. Current and voltage records were filtered at 0.3 kHz. We used holding potentials of -38 to -50 mV and hyperpolarizing commands of 5–20 mV and durations of 1 s. Current and voltage data were acquired by D/A sampling and acquisition software (P-Clamp; Axon Instruments); most often, -5 , -10 , -15 and -20 mV voltage commands were applied in sequence five times for subsequent averaging. Current measures thus were derived from averages of five sweeps at each of the four voltage commands. I_M amplitude was measured with software (Clampfit, Axon) that fitted exponential curves (1 exponent, R values = 0.85 – 0.99) to the I_M relaxation by two methods: (1) curve fitting to the peak of the initial (quasi-instantaneous) current that usually fell within 15–25 ms of step onset; or (2) exponential fitting through the peak with extrapolation back to command onset. These methods gave essentially equivalent results with respect to relative effects of drugs, although the extrapolated amplitude was obviously bigger (10–25%; average 20%) than amplitude at peak. For statistical purposes the first (peak) method was used. Baseline (holding) current and relative steady state conductance were also measured by the same software.

indomethacin never reduced increases in I_M ¹⁵ elicited by arachidonic acid. Superfusion of prostaglandins E_2 , $\text{F}_{2\alpha}$ or I_2 (2 – $60 \mu\text{M}$) did not significantly alter I_M (seven cells; data not shown). Taken together, these findings suggest that a cyclooxygenase product of arachidonic acid is probably not involved in the somatostatin effect on I_M .

Superfusion of nordihydroguaiaretic acid (NDGA, $10 \mu\text{M}$) a lipoxygenase inhibitor¹⁶, abolished both the somatostatin-induced (five of six cells; Fig. 2*e*) and the arachidonic acid-induced (11 of 11 cells; Figs 2*d* and 3*a*) I_M augmentation, bringing the baseline I_M back to control levels. NDGA superfused alone had no significant effect on I_M in four additional cells. Furthermore, long superfusion times of somatostatin alone (up to 25 min) did not decrease the somatostatin-induced effect on I_M .

Several lipoxygenase products are candidates for mediation of somatostatin effects. Superfusion of the 5-lipoxygenase product leukotriene C_4 (LTC_4 ; 5 – $8 \mu\text{M}$), even in the presence of NDGA, mimicked the action of somatostatin and arachidonic acid in augmenting both the M -current and input conductance, and in eliciting an outward baseline current, in four of five cells (Figs 3*a, b*). Leukotriene B_4 (15 – $30 \mu\text{M}$) had no significant effect on these parameters (four cells; Fig. 3*c*). In preliminary studies, both 5,6-methanoleukotriene A_4 methyl ester ($10 \mu\text{M}$) and

FIG. 2 *a*, Current-voltage curve constructed from the instantaneous (filled symbols) and steady state currents (open symbols) for two different cells before (control: triangles) and during (diamonds) superfusion of either SS14 (left panel) or arachidonic acid (right panel). Both experiments in the presence of 2 mM Cs⁺ to block *I*_Q. Arrows indicate the reversal potential for *I*_M (−73 mV for the left and −79 mV for the right cell), which are very similar to those previously reported for hippocampal pyramidal neurons¹², and are not significantly changed by either SS14 or arachidonic acid. Resting potentials were −66 mV (left cell) and −68 mV (right cell). *b*, Curves from the same cells showing the changes in the M conductance (ΔG_M) underlying the current relaxation (closing of M-channels), calculated by dividing the amplitude of relaxation (inverted across the range of voltage steps) by its driving force (holding potential minus reversal potential). Note that the conductance is nearly doubled by SS14 (top panel) or arachidonic acid (lower panel), compared to controls (Cont.). *c*, Average *I*_M data ($\pm$ s.e.m.) from five cells tested with indomethacin (Indo; 8–10 μ M) plus arachidonic acid (ArAc; 120–150 μ M): addition of indomethacin actually increases *I*_M beyond the augmentation elicited by arachidonic acid alone. *d*, Average data from 11 cells tested with arachidonic acid plus NDGA. NDGA (10 μ M) significantly ($P < 0.01$) blocks the *I*_M increasing effects of arachidonic acid (120–150 μ M) (see Fig. 3a for digitized record of NDGA block of arachidonic acid). *e*, NDGA (10 μ M) significantly ($P < 0.01$) blocks effects of SS14: average data from six cells. In *c* and *e*, individual standard error bars removed for clarity; s.e.m. averaged from all the data is 19.3 pA for *c* and 20.8 pA for *e*.



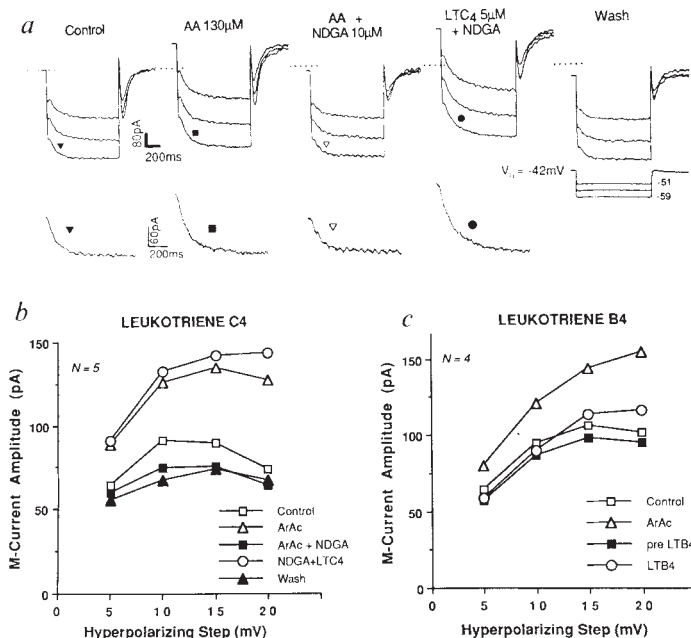
5,6-dehydroarachidonic acid (10 μ M), specific 5-lipoxygenase inhibitors¹⁷, completely blocked the M-current augmenting effects of somatostatin (three cells) and arachidonic acid (one cell).

Augmentation of *I*_M by cyclic AMP was recently reported for smooth muscle⁵. In our present studies of hippocampal neurons, however, superfusion of cyclic nucleotides (8-bromo cyclic GMP; 8-bromo cyclic AMP; dibutyl cyclic AMP) had no consistent effect on the amplitude of *I*_M at concentrations up to 2.5 mM, although occasional cells showed a small increase.

Thus, our combined data suggest that some lipoxygenase metabolite of arachidonic acid may be a second messenger or mediator for the *I*_M-augmenting action of somatostatins in hippocampal pyramidal neurons, and therefore also for the somatostatin-induced hyperpolarizations⁴. The actual metabolite or metabolites involved in hippocampal somatostatin

effects is not known, although our data with LTC₄ suggests that a leukotriene is the best candidate. LTC₄ can be metabolized further to LTD₄ and LTE₄, which might also be involved. Our preliminary findings that specific 5-lipoxygenase inhibitors antagonize both somatostatin and arachidonic acid-induced *I*_M effects are consistent with leukotriene mediation of somatostatin effects. As far as we know, this is the first electrophysiological evidence for such eicosanoid mediation of a transmitter effect in a vertebrate central neuron. Prostaglandins have been implicated in mediation of the biochemical effects of vasoactive intestinal peptide in brain slices¹⁸, and lipoxygenase products may regulate this peptide's release¹⁹. Piomelli *et al.* have shown that 12-lipoxygenase metabolites may mediate FMRFamide effects on a K⁺ current⁸ and hyperpolarizations elicited by histamine²⁰ in *Aplysia* neurons. In addition, leukotrienes have been shown to alter spontaneous extracellular firing rate in rat cerebellar Purkinje cells *in vivo*²¹.

FIG. 3 LTC₄, but not LTB₄, mimics arachidonic acid (ArAc) and somatostatin effects on *I*_M. *a*, Top row: digitized current records from a CA1 pyramidal neuron. Arachidonic acid superfusion (130 μ M) augments *I*_M, increases conductance and causes an outward baseline current. Superfusion of NDGA 10 μ M (with arachidonic acid) completely reverses these effects. Subsequent superfusion of LTC₄ (5 μ M) in the continued presence of NDGA again augments *I*_M. Washout brings all current measures back to near-normal levels. Lower row: single *I*_M relaxations isolated from the upper current traces (with matched symbols) and magnified for clarity. *b*, Average of *I*_M amplitudes for five cells tested with LTC₄, using holding potentials of about −45 mV, and the same drug protocol as in *a*. Average *I*_M is significantly increased ($P < 0.05$) by LTC₄. *c*, Average *I*_M amplitude from four other cells tested with LTB₄ (15–30 μ M), which has no significant effect on *I*_M ($P > 0.1$). 'Pre-LTB₄' is the 12–20 min period between arachidonic acid and LTB₄ superfusions for washout of ArAc (two cells) or arachidonic acid blockade with NDGA 10 μ M (two cells). In *b* and *c* standard error bars removed for clarity; average s.e.m. from all the data is 19.6 pA for *b* and 8.5 pA for *c*.



To our knowledge there are no reports showing biochemical evidence of somatostatin-induced increases in PLA₂ or lipoxygenase activity. It is possible that a GTP-binding protein may be involved in activation of PLA₂ (ref. 22). Leukotrienes are synthesized in several regions of the brain, including the hippocampus²³. Specific LTC₄ binding sites are also found in several brain regions and are among the most numerous in the hippocampus; binding sites for LTB₄ and LTE₄ are weak and those for LTD₄ undetectable²⁴. The exact subcellular location of receptors for the lipids however, is still unknown: given the great membrane permeability of the lipids, they could act on the

M-channel by either an intracellular or extracellular route, as suggested by others^{8,25,26}. Thus, presynaptic or feedback effects cannot be excluded. Although the exact physiological role and site of action of the relevant lipid(s) cannot be stated at this time, it seems likely that, as in some invertebrate neurons^{8,20}, a single ion channel type in mammalian central neurons can be regulated in opposing directions by two different ligands (somatostatin and acetylcholine) acting through different second messenger systems. Such bidirectional regulating mechanisms should provide sensitive biasing of ion channel activity, and therefore neuronal excitability. □

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Dual-component NMDA receptor currents at a single central synapse

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PRESENT thinking about the way that the NMDA (N-methyl-D-aspartate) class of glutamate receptor operates at central synapses relies mainly on information obtained from single-channel and whole-cell recordings from cultured neurons stimulated by exogenous NMDA receptor agonists^{1,2}. The mechanisms that operate in the postsynaptic membrane of a normal neuron following release of the natural transmitter are far less clear. An important problem is that most normal neurons receive many excitatory synapses (10³–10⁵ per cell) and these synapses are located on slender dendritic elements far away from the somatic recording site, making the study of discrete synaptic events difficult. Typically, when populations of synapses are activated, NMDA receptor-mediated synaptic potentials appear as slowly rising, long-lasting waves superimposed on faster, non-NMDA-receptor potentials^{2–8}. Although believed to be critical for NMDA receptor function^{9,10}, this slow time-course would not be predicted from single-channel kinetics and its origin remains puzzling. We have now analysed the events occurring at the level of a single excitatory synapse using a simple, small, neuron—the cerebellar granule cell—which has an unusually simple glutamatergic⁸ input. By applying high-resolution whole-cell recording techniques to these cells *in situ*¹¹, we were able to study the nature of elementary NMDA receptor-mediated synaptic currents. Contrary to expectations, the prominent currents are fast but are followed by slow ones. Both types of current are strongly voltage-dependent but differ subtly in this

respect. Furthermore, the currents are absent unless glycine is provided.

Cerebellar granule cells are only about 6 µm in diameter and receive a simple monosynaptic excitatory input from mossy fibres, which originate mainly in nuclei in the brain stem and spinal cord. The total number of excitatory synaptic boutons supplied to each cell amounts to only 3 or 4, one on each short dendrite^{12,13}. Granule cells near the surface of parasagittally cut rat cerebellar slices were selected for study. One or two of the dendrites of these more superficial cells would have been cut off, leaving one or two synapses buried within the slice (Fig. 1a). The patch electrodes contained Nystatin to obtain 'perforated' whole-cell recordings, which minimize loss of cytoplasmic components¹⁴, and CsCl as the main electrolyte. All bathing solutions contained tetrodotoxin and bicuculline to block action potentials and inhibitory currents. The membrane capacitance was 3.3 ± 0.37 pF (mean ± s.d. of 87 cells in 11 slices) which, assuming a specific capacitance of 1 µF cm⁻², gives an equivalent mean spherical cell diameter of just 10 µm.

The most obvious currents were fast and of a shape suggesting them to be synaptic currents produced by spontaneous release of quanta of transmitter from presynaptic terminals^{15,16}. Consistent with this interpretation, their frequency was increased by perfusion of K⁺ to depolarize the nerve terminals (Fig. 1c) and their amplitude distribution was bell-shaped (data not shown). The currents were identified as NMDA receptor-mediated on the basis of the following observations.

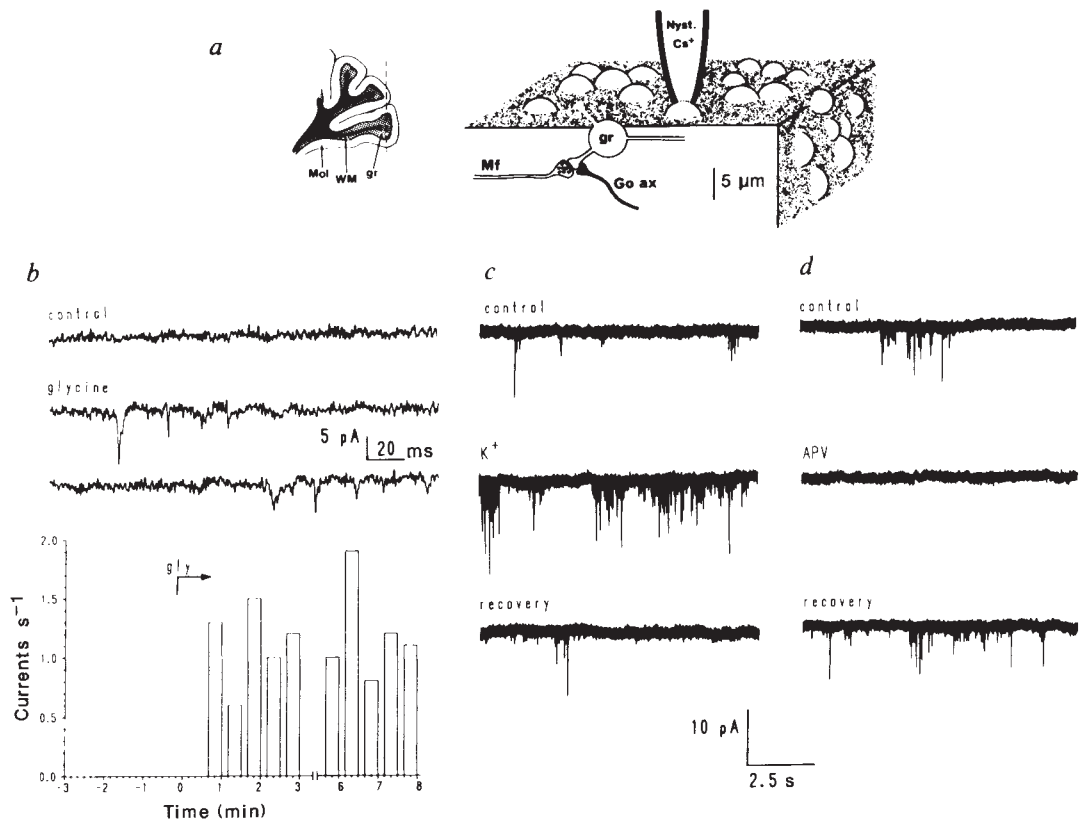
First, the currents were absent unless glycine was supplied (Fig. 1b). Low concentrations of glycine augment responses to NMDA by binding to a high-affinity site on the NMDA receptor¹⁷. In cultured neurons, fast perfusion techniques are needed to prevent activation of this site by glycine continuously released from nearby cells. In our initial tests, the incubation bath (1.5 ml capacity containing 3–5 slices) was left static for 0.5–3 h before recording. Nevertheless, all 17 cells studied (4 slices) were initially silent. But 14 of them displayed spontaneous currents on local addition of glycine. With 10 µM glycine in a continuously perfused medium (subsequently adopted), 70% of recorded cells displayed spontaneous currents.

Second, the currents were reversibly abolished by the NMDA antagonist APV (D-2-amino-5-phosphonovaleate; Fig. 1d), but

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FIG. 1 Spontaneous synaptic activity is revealed by addition of glycine (*b*) and is enhanced by K^+ (*c*) and abolished by APV (*d*). Whole-cell recordings were made from granule cells in thin slices of rat cerebellum (shown schematically in *a*) but no activity could be observed, in this case recording over 10 min with the slice bathed for 2 h in nominally Mg^{2+} -free medium, until glycine was delivered from a pipette (containing the amino acid at a concentration of $10 \mu M$) positioned 50–100 μm from the recorded cell. In a different experiment, K^+ and APV were also perfused locally (30 mM and 100 μM in the pipette, respectively) with $10 \mu M$ glycine and 1.2 mM Mg^{2+} in the bath. The effect of APV was observed all five cells tested and, with K^+ , quantitation of current frequency (currents $\cdot s^{-1}$) showed a significant increase (2- to 16-fold; three cells) within the first 30 s of application. The holding potential was -40 mV in all cases and the currents were filtered at 2 kHz. The currents were unaffected when $10 \mu M$ strychnine, an antagonist acting at inhibitory glycine receptors, was perfused together with glycine (six cells, two slices; see also Fig. 3 legend). *a*, Left: Mol, molecular layer; WM, white matter; gr, granule cell layer. *a*, Right: Mf, mossy fibre; gr, granule cell; Go ax, Golgi cell axon.



METHODS. Thin (70–120 μm) parasagittal slices of cerebellar vermis from young (12–15-day-old) rats were prepared using a Vibroslice¹¹. A microknife cut was made across the upper part of the granule cell layer (see Fig. 1*a*) to improve visibility (Olympus CK2 inverted microscope equipped with phase contrast optics) and sever the long granule cell axons. The slices were transferred to a polylysine-coated Petri dish containing 1.5 ml of a solution maintained at 30 °C and containing NaCl (130 mM), KCl (3 mM), Na_2HPO_4 (1.2 mM), $CaCl_2$ (2 mM), $MgSO_4$ (1.2 mM), glucose (11 mM), Tris-HCl (15 mM)

were unaffected by CNQX (6-cyano-2,3-dihydroxy-7-nitroquinoxaline) which is a relatively selective non-NMDA antagonist in the presence of $10 \mu M$ glycine¹⁸ (see Fig. 3*b*). Third, with a physiological Mg^{2+} concentration (1.2 mM) in the bathing solution, current-voltage relationships (Fig. 3*a*) were linear ($R = 0.99$) from positive holdings to -20 mV, reversed at -6.5 mV (uncorrected value; see Fig. 3 legend) and, as is characteristic of NMDA currents^{1,2}, displayed a negative slope conductance between -30 and -70 mV. In the absence of added Mg^{2+} , the curve was linear from positive potentials down to -40 mV and the currents thereafter were larger than with Mg^{2+} added (Fig. 3*a*), consistent with a block of the NMDA channels by this ion and the presence of residual Mg^{2+} in the nominally Mg^{2+} -free extracellular solution. Over the linear range, the slope was 255 pS (1.2 mM Mg^{2+}), 254 pS (no added Mg^{2+}) and 246 pS (1.2 mM Mg^{2+} , $10 \mu M$ CNQX). Assuming a single channel conductance of 50 pS^{1,2}, the fast currents are composed, on average, of the synchronous opening of five NMDA channels.

Kinetic analysis of these currents resulted in mean rise times (measured between 20–80% of the peak; means $\pm$ s.d.) of 0.57 ± 0.65 ms at -60 mV and 0.77 ± 0.55 ms at $+50$ mV ($n = 10$). The corresponding times to peak were 0.77 ± 0.65 ms and 1.13 ± 0.55 ms. These rapid kinetics are reminiscent of those of spontaneous endplate currents recorded at the neuromuscular junction¹⁵. The decay of the individual currents, however, was

(pH 7.4), tetrodotoxin ($1 \mu M$) and bicuculline ($10 \mu M$). In some experiments, $MgSO_4$ was omitted. The slices were immobilized by nylon threads fixed to a silver ring. A stream of prewarmed solution was gently superfused from a local pipette over a selected folium for 10–30 min. Except in the earlier experiments (see text) the bathing medium was continuously exchanged (1 ml min^{-1}). The recording microelectrodes (10 – 12 M Ω) were filled with a solution containing Nystatin ($100 \mu g$ ml⁻¹) and CsCl (140 mM), NaCl (4 mM), $CaCl_2$ (0.5 mM), EGTA (5 mM), HEPES buffer (5 mM), pH 7.2 (CsOH). The Nystatin method was adopted both for theoretical reasons¹⁴ and because the success rate in gaining access to the cell interior was considerably higher than with conventional whole-cell recording. Whole-cell currents were derived using a List EPC7 amplifier. The access resistance averaged 70 M Ω and the steady-state input resistance was typically 10 G Ω .

complex, as expected from the random closure of so few NMDA channels. Digital averaging (Fig. 2*b*) suggested a decay time constant (τ_d) at $+50$ mV of 7.1 ± 0.9 ms (mean from best exponential fits of the averaged current decay in three cells $\pm$ s.d.). This value is in good agreement with the reported mean lifetime of unblocked NMDA channels^{19,20}. The decay became faster at negative potentials (Fig. 2*b*); at -50 mV, τ_d was 3.1 ± 1.1 ms ($P < 0.02$). This could reflect a reduced channel-burst duration at negative membrane potentials.

Beyond the main peak, the kinetics of single currents were not easily resolved. This was because the rapid events were often followed by a noisy tail, highly variable in duration, but frequently lasting for tens of milliseconds (Fig. 2*a*). In averaged records, these tails generated a 'hump', peaking 20–25 ms after the start of the fast component (Fig. 2*b*). The current-voltage plot of the hump over the range $+50$ to -70 mV was like that of the fast current (Fig. 3*c*), but between -70 and -80 , the fast current increased whereas the hump decreased. The ratios of the currents at these two potentials were 1.90 ± 0.31 for the fast component and 0.84 ± 0.29 for the hump (means $\pm$ s.d.; data from Fig. 3*a* and *c*; $P < 0.002$).

It can be concluded that quanta of excitatory transmitter are released spontaneously at this central synapse and that they elicit postsynaptic currents exclusively through NMDA receptors. It is likely that other synapses are similar in this respect

as 'tonic' NMDA receptor activation has been detected as a background current in hippocampal neurons in the absence of synaptic stimulation²¹. Such spontaneous activity could have an important influence on neuronal excitability²¹. Non-NMDA receptors are present on granule cells and can be activated following electrical stimulation of mossy fibres⁸, but they may need a higher concentration of transmitter to become effective¹⁹.

The primary (fast) synaptic current elicited through NMDA receptors has largely predictable kinetics, assuming quanta of transmitter bind to receptors located close to the release site and so cause the synchronous opening of about five NMDA channels. The subsequent slow current, however, is likely to be the one responsible for the slow synaptic NMDA potentials previously detected in this and several other pathways following electrical stimulation of afferent fibres²⁻⁸. We can now exclude several hypotheses that have attempted to explain the slow time-course of this component by invoking network-related factors, such as polysynaptic pathways². Instead, an attractive possibility is that after the channels mediating the fast component have closed, the transmitter dissociates and then rebinds to receptors asynchronously²². Given the delay to the peak of the slow wave, the operative receptors could be located some distance away, perhaps extrasynaptically. One prediction of this hypothesis is that the time to the peak of the slow wave should shorten as the decay rate of the fast currents increases, as indeed the averaged records (Fig. 2b) suggest.

Finally, the precise action of glycine and whether the ambient levels of glycine are limiting for NMDA receptor function or not, are both controversial issues²³. Our results strongly suggest that glycine is an obligatory co-agonist at the synaptic NMDA receptor²⁴, as no currents could be detected in the absence of added glycine. This, in turn, implies that the ambient levels in the synaptic cleft are very low, probably ≤ 30 nM (from glycine dose-response curves²⁵). Although it cannot be excluded that diffusion of glycine out of the slice is responsible, our findings with preparations bathed for long periods in unstirred solutions would not favour this possibility. Moreover, the availability of glycine is limiting for NMDA receptor function in the cerebellum *in vivo*²⁶. Thus, it is more likely that there is a mechanism

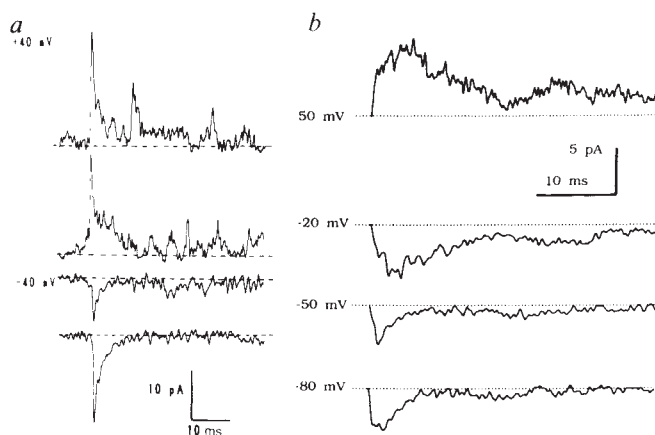


FIG. 2 Properties of spontaneous currents. *a*, Individual currents recorded at +40 and -40 mV (1.5-kHz low-pass filter). Note the 'noise' appearing after the decay of the initial fast current in the upper three traces. When averaged (*b*) this noise generates a hump. Note also in *b* that the decay of the fast component and the time to peak of the hump seem to be shortened by hyperpolarization and that the voltage dependence of the peak of the fast component differs from that of the hump (compare -50 and -80 mV). The perfusing solution contained 10 μ M glycine, 1.2 mM Mg^{2+} and (in *b*), 10 μ M CNQX as well. For each record in *b* 8–15 single currents were digitized at 20 kHz and averaged off-line by synchronizing and zeroing them at the start of the fast current. All events having an amplitude equal to, or greater than, twice the background noise were included in the averages. The data are from the same cell except for that at +50 mV.

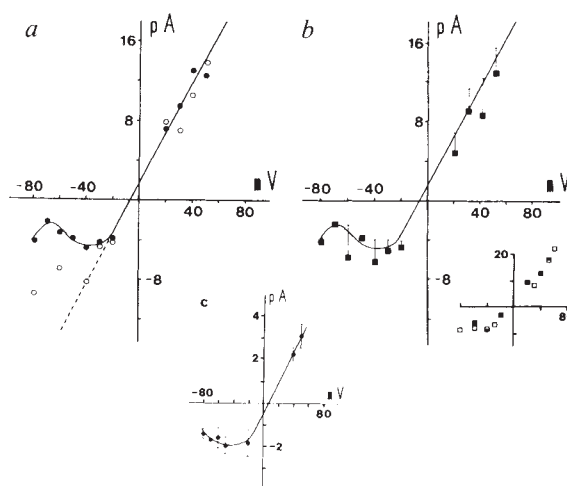


FIG. 3 Mean current-voltage (*I-V*) relationships for the fast current (*a*, *b*) and the hump (*c*). In *a* the bathing solution contained either 1.2 mM Mg^{2+} (filled circles) or no added Mg^{2+} (open circles). Standard deviations are omitted for clarity but averaged 24% of the means. The data in *b* ($\pm$ s.d.) were from cells incubated with 1.2 mM Mg^{2+} and 10 μ M CNQX. Each point (*a* and *b*) represents the mean peak amplitude of individual currents in five cells (two different slices). All currents of amplitude greater than or equal to twice background noise were included in the analysis; the mean current at -70 mV in Mg^{2+} -containing solution may thus be an overestimate (noise level, 1–1.5 pA). The current amplitudes were not significantly affected by strychnine; for example, at +40 mV, with no added Mg^{2+} and 10 μ M strychnine in the perfusing solution, the mean current in three cells ($\pm$ s.d.) was 8.4 ± 2.2 pA. The inset (*b*) shows the *I-V* curve for a single cell in the presence (solid symbols) and absence (open symbols) of 10 μ M CNQX. The *I-V* curve for the hump (*c*) was obtained from measurements of peak amplitude in averaged currents (Fig. 2b) from four different cells (means $\pm$ s.d.). The solid line (*a* and *b*) fits data points in 1.2 mM Mg^{2+} , zero CNQX (by linear regression at potentials above -20 mV, the remainder by eye). The apparent reversal potential for these currents is -6.5 mV. In the perforated patch whole-cell recording configuration, a Donnan equilibrium will develop between the pipette and the cytoplasm, which should make the pipette 8.9 mV positive¹⁴ and thus shift *I-V* relationships to the left by this value. As the reversal potential of the NMDA current (considering only the Nystatin-permeant ions in the pipette and in the bath) can be calculated to be +4.3 mV (ref. 29), the synaptic currents should theoretically reverse at -4.6 mV, which is in reasonable agreement with the experimental value.

maintaining glycine at a low concentration in this synapse. The axon terminals of Golgi cells are equipped with a high-affinity glycine carrier²⁷ and are in an anatomically appropriate position to undertake this function. Being rich in glycine²⁸ the same terminals could supply the amino acid to the synapse under appropriate conditions and so regulate granule cell NMDA currents. □

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Linkage of a nasopharyngeal carcinoma susceptibility locus to the HLA region

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THE frequency of nasopharyngeal carcinoma is nearly 100-fold higher in southern Chinese than in most European populations¹. Earlier studies have suggested that an increased risk of nasopharyngeal carcinoma is associated with specific haplotypes in the HLA region: relative risks slightly over twofold were found for haplotypes A2, Bw46 and the antigen B17 (refs 2–4). We now report a linkage study based on affected sib pairs which suggests that a gene closely linked to the HLA locus confers a greatly increased risk of nasopharyngeal carcinoma. The maximum likelihood estimate is of a relative risk of approximately 21. The relationship between this suspected disease susceptibility gene (or genes) and known viral and environmental aetiological factors remains to be elucidated.

Our approach was to identify sibships that had more than one individual affected with nasopharyngeal carcinoma (NPC), and for which sufficient individuals could be typed to permit

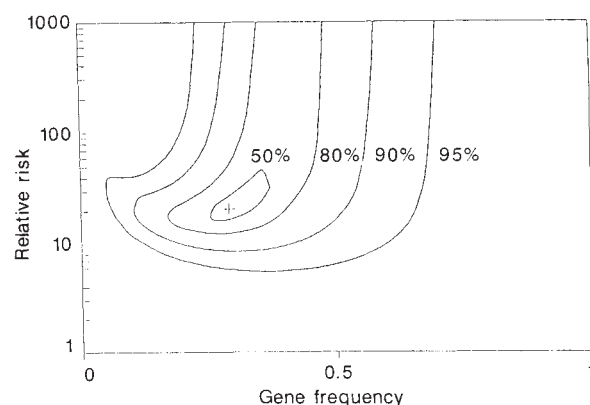


FIG. 1 For the model fitting, inference was based on the log-likelihood difference conditional on the disease phenotypes, or lod score. The use of this conditional likelihood allows inferences to be made free from ascertainment bias^{6,8}. Likelihood calculations were performed using the program LINKAGE⁷ for single-gene dominant and recessive models, varying the gene frequency relative risk associated with the disease susceptibility gene, and the recombination fraction θ between the susceptibility gene and the HLA region. In particular, the lower 95% confidence limit for the relative risk associated with the susceptibility gene, r_0 , is given by:

$$2 \left[\max_{r, \theta} l(r, f, \theta) - \max_{r, \theta} l(r_0, f, \theta) \right] = 3.84$$

where $l(r, f, \theta)$ denotes the log-likelihood function and 3.84 is the upper 95% point for a χ^2 distribution on 1 degree of freedom. The contours shown define confidence regions for r and f , at the 95%, 90%, 80% and 50% level with $\theta = 0$. The absolute maximum of the likelihood occurs when $\theta = 0$, with values of $f = 0.29$, $r = 20.9$, and is marked by a cross (+).

unambiguous assignment of haplotypes⁵. The study began in Singapore and Hong Kong in 1976 and extended in 1983 to Nanning in the Guangxi Autonomous region of the People's Republic of China, where more sib pairs were accessible (see Table 1 legend). In each area, the members of each sibship, their parents and children were visited and blood samples taken. Thirty-four sibships with more than one case of NPC were identified, 31 with two cases and the remainder with three cases (Table 1). Of the sib-pair families, four had to be excluded, one because the pair were twins of unknown zygosity (but HLA identical) and three because an excess of haplotypes was seen in the family and parentage was ambiguous.

HLA typing on the samples from Hong Kong and Singapore was performed in Singapore and restricted to locus A and B antigens, using a panel of over 200 antisera of Chinese, Malay, Japanese, Filipino and Caucasian origin. HLA typing in Nanning used a panel of 143 allelic typing antisera (from Saint Louis Hospital, Paris) corresponding to HLA A, B, C and in most cases DR loci. Of the 38 NPC cases tested, the most common DR antigens were DR2 (18/38) and DR4 (16/38). No

TABLE 1 Sib-pair analysis

	2 Expected under				1 Expected under				0 Expected under			
	Observed	No linkage	Dominant	Recessive	Observed	No linkage	Dominant	Recessive	Observed	No linkage	Dominant	Recessive
Sib pairs*	16	6.75	10.83	13.96	8	13.50	13.18	10.40	3	6.75	2.99	2.64
Triplet†	2	2.25	3.30	4.01	4	4.50	4.39	3.91	3	2.25	1.31	1.08
Total	18	9.00	14.13	17.97	12	18.00	17.57	14.31	6	9.00	4.30	3.72

Number of sib pairs sharing two, one or zero HLA haplotypes identical by descent, together with the expected numbers under the null hypothesis of no linkage, and under the best fitting dominant and recessive models. For the sib trios, all possible sib pairs are considered.

Goodness of fit¹¹ of the hypothesis of no linkage, χ^2 (2 d.f.) for sib pairs only = 17.0P < 0.001; for all pairs (both sib pairs and triples) = 12.0P < 0.005. Goodness-of-fit tests of the recessive and dominant models are each given by a 1 d.f. likelihood ratio test against a three-parameter model, with relative risk r_1 for the DS heterozygote, and r_2 for the DS homozygote. χ^2_1 for recessive model = 0.32; χ^2_1 for dominant model = 2.95 (P = 0.08).

* 18 from China, 3 from Hong Kong, 5 from Singapore and 1 from Malaysia.

† 2 from China and 1 from Hong Kong.

other DR antigen was present on more than seven individual cases.

The haplotype similarity of affected sibs and triplets is shown in Table 1, together with the expected numbers if no linkage were present. The total data show a clear divergence between observed and expected ($P < 0.005$). Among the sib pairs, comparing observed with expected gives a χ^2 value of 17.0 (2 d.f.). This highly significant divergence demonstrates the existence of NPC disease susceptibility gene(s) linked to the HLA region.

For linkage analysis, single-gene models were explored⁶⁻⁸, and as models assuming dominant inheritance fitted poorly (see Table 1), the subsequent analyses were based on recessive models. Figure 1 shows the likelihood of the observed results (lod score) for varying values of the relative risk (r) and the gene frequency (f) of the putative disease susceptibility gene when the recombination fraction (θ) is zero. The maximum lod score under the recessive model is +2.39, and under the dominant model +1.68, with P values of 0.004, and 0.024 respectively.

The maximum likelihood estimate occurs when $\theta = 0$, $r = 20.9$ and $f = 0.29$. Infinite values of r are only slightly less likely for a range of values of f ; the minimum value of r consistent with the data is 5.1 at the 95% level, and 9.8 at the 90% level. With values of θ greater than zero, all the likelihood contours are further removed from the axis given by $r = 1$.

These results show the existence of an NPC disease susceptibility gene(s) conferring an increased risk for NPC some tenfold

larger than that associated with Bw46 or B17, with the upper 90% confidence bound being infinite. The results thus provide strong support for the original hypothesis⁵ that much of the high risk for NPC among the southern Chinese is due to HLA-associated genes. The lifetime risk of NPC among Cantonese males in Singapore¹ is 1.6%, indicating that the frequency of a recessive disease susceptibility gene should be at least 0.13.

The mechanism by which an HLA-linked disease susceptibility gene might act is unknown. Two proposed environmental aetiological agents for NPC are the Epstein-Barr virus (EBV) and some constituents of preserved foods, including salted fish⁸⁻¹⁰. Insight into the mechanism of action of NPC disease susceptibility gene(s) should help to clarify the respective role of EBV, environmental cofactors and genetic susceptibility in the development of NPC. □

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Residues of the variable region of the T-cell-receptor β -chain that interact with *S. aureus* toxin superantigens

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THE $\alpha\beta$ T-cell antigen receptor (TCR) recognizes antigenic peptides in the context of self major histocompatibility complex (MHC) molecules¹⁻⁴. The specificity of recognition of MHC plus antigen is generally determined by a combination of the variable elements of α - and β -chains of the TCR⁵⁻⁸. Several types of antigen, however, have been identified that, when bound to MHC molecules, stimulate T cells bearing particular variable-region β -chain (V β) elements irrespective of the other variable components of the TCR⁹⁻²¹. These have been termed 'superantigens', and here we are concerned with one type of superantigen, the toxins produced by *Staphylococcus aureus*. T cells have been found that bear closely related members of the same V β family but respond differently to *S. aureus* toxins^{15,19,22}; in particular, cells bearing the human V β 13.2 element respond to toxin SEC2, whereas cells bearing human V β 13.1 do not. We have now defined the residues of the V β element responsible for this difference, and find that they reside in a region thought to lie on the side of the TCR molecule, away from the conventional antigen/MHC-binding site. The evolutionary conservation of this site may be due to its having an important role in some function of the TCR other than the binding of conventional antigen plus MHC.

For these structure-function studies, we designed a trans-

fection system in which the products of human V β genes were introduced into an otherwise murine TCR expressed on the surface of a mouse T-cell hybridoma (see legend to Fig. 1). The specificity of this chimaeric receptor could then be determined by the ability of the transfectants to produce interleukin-2 (IL-2) when stimulated with various toxins presented by cells bearing class II MHC molecules. Initially, transfectants were produced with receptors bearing either human (h) V β 13.1 or V β 13.2. Representative clones with good TCR surface expression were tested for reactivity to toxins presented either by A20, an H-2^d class II⁺ murine B-cell lymphoma (Table 1), or by murine splenic class II⁺ non-T cells of various H-2 haplotypes (Table 2). Immobilized anti-C β antibody (that is, an antibody to the TCR β -chain constant residues) was used as a positive control to measure maximal TCR-mediated stimulation. The V β 13.1-bearing transfectant failed to respond to any of the toxins presented by any of the murine presenting cells, although it responded well to immobilized anti-C β antibody. The V β 13.2 transfectant responded to the toxin SEC2 and less well to SEC3 regardless of the presenting cells. These differences in reactivity were seen over a range of toxin concentrations (data not shown).

These results establish several points. First, they show that human-murine chimaeric β -chains assembled properly with murine α -chains and murine CD3 to yield functional receptors. Second, the specificities of the human V β chimaeric receptors are consistent with those seen in normal human T-cells¹⁹. Finally, for an individual TCR, interaction with the allelic residues in the MHC portion of the toxin-MHC ligand can influence the degree of response^{15,23}. The finding that T cells bearing V β 13.1 and V β 13.2 respond differently to the toxins SEC2 and SEC3 regardless of the allelic form of murine class II used for presentation, suggests that the residues that differ between these two V β regions are involved in toxin rather than MHC interaction.

The amino-acid differences between V β 13.1 and V β 13.2 are shown in Fig. 2 superimposed on a schematic drawing of a portion of the structure of V β , predicted by Chothia *et al.* on the basis of the conservation of critical residues between V β and the variable region of the immunoglobulin heavy chain, VH²⁴. There are two main stretches of amino acids at which the two V β regions differ. One is predicted to lie in the second complementarity determining region, CDR2—part of the

TABLE 1 Response of transfected T-cell hybridomas to *S. aureus* toxins

V β -transfected T-cell hybridoma	$\alpha\beta$ TCR density*	Units ml ⁻¹ IL-2 produced in response to A20-11.1 and <i>S. aureus</i> toxins										Anti-TCR
		None	SEA	SEB	SEC1	SEC2	SEC3	SED	SEE	ExT	TSST-1	
hV β 13.1-1	34	—†	—	—	—	—	—	—	—	—	—	843
hV β 13.2-1	19	—	—	—	—	86	44	—	—	—	—	256
hV β 13.1D2-1	30	—	—	—	—	678	200	—	—	—	—	725

Three T-cell hybridomas bearing different hV β regions were tested for their ability to respond with IL-2 production to either nine different *S. aureus* toxins presented by a murine H-2^d B-cell lymphoma (A20-11.1) or an immobilized anti-TCR antibody (H57-597), as previously described²⁵. The limit of detection was 10 U IL-2 ml⁻¹. T-cell hybridomas hV β 13.1-1, hV β 13.2-1 and hV β 13.1D2-1 express hV β 13.1, hV β 13.2 and hV β 13.1D2 respectively. The concentrations of toxins used were: 10 μ g ml⁻¹ for SEB, SEC1, SEC2, SEC3 and SED; 1 μ g ml⁻¹ for SEA, ExT and TSST-1.

* $\alpha\beta$ TCR receptor density is presented as mean fluorescence of T cells stained with biotinylated H57-597 plus phycoerythrin-streptavidin.

† <10 U IL-2 ml⁻¹ indicated by —.

receptor thought to be responsible for binding ligands of conventional antigenic peptides complexed to MHC. The other stretch of differences lies in a region of the β -chain that, on the basis of the immunoglobulin model, would form part of the side of the β -chain, exposed to the aqueous phase and well away from the peptide antigen/MHC-binding site or the interaction points between V β and V α . We decided to concentrate on this part of V β , partly because amino-acid residues in it contribute to the interaction between V β and the mouse endogenous superantigen, Mls-1^a (ref. 20). Therefore, we prepared a third transfectant, hV β 13.1D2, in which the unique residues of V β 13.2 from positions 67–77 were introduced into V β 13.1.

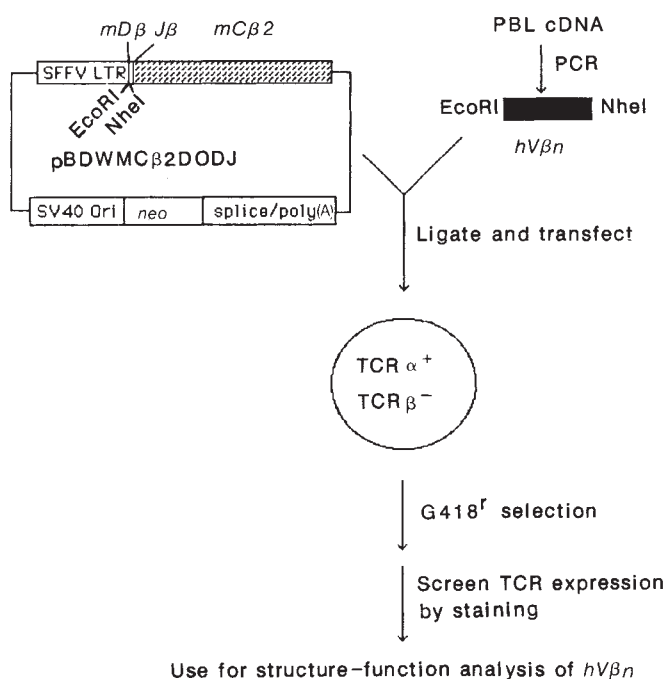
The transfectant bearing this mosaic V β showed the same pattern of reactivity to the panel of toxins and presenting cells as that seen with the V β 13.2 transfectant, that is, high reactivity to both SEC2 and SEC3, but little or none to the other toxins, regardless of the H-2 type of the presenting cell (Tables 1 and 2). The magnitudes of the responses of the transfectant bearing the mosaic V β were in fact higher than those seen with V β 13.2 even when anti-C β antibody was used as the stimulant. This was probably due to a higher density of receptors on the mosaic V β transfectant than on the other cell lines.

Therefore, the introduction of eight amino-acid residues at positions 67–77 of human V β 13.2 into human V β 13.1 is sufficient to confer the toxin reactivity of hV β 13.2 on the mosaic V β produced, suggesting that the site for toxin-binding on V β lies in this region of the molecule, not in the face containing the predicted complementarity-determining regions thought to control conventional peptide antigen recognition. In addition, the fact that this same region of V β has been implicated in the binding of an endogenous mouse superantigen, Mls-1^a, to the TCR (ref. 20) suggests that the bacterial toxin and endogenous murine superantigens bind to the same or related sites on V β .

None of the structures of the endogenous superantigens are known; the discovery of overlapping binding sites on V β may indicate that the endogenous superantigens are indeed proteins, with structures related to those of the toxins. The evolutionary conservation of this superantigen binding site on V β is puzzling, as it both offers a target for the bacterial toxins and results in large scale deletion of T cells bearing particular V β elements in individuals carrying an endogenous superantigen, although apparently not contributing to recognition of conventional antigen-MHC ligands^{9–20}. This site may play an indispensable part in some other TCR function, such as the positive selection of

FIG. 1 Scheme for expression of hV β elements on murine T-hybridoma cells. The studies used an expression vector/host-cell system wherein specific human V β -containing constructs were expressed on the surface of a murine T-hybridoma cell line. In brief, an expression vector was constructed that carried the mouse C β 2 gene and mouse D β J β elements (pBDWMC β 2DODJ). A human V β region sequence was prepared by amplification of cDNA prepared from human peripheral blood lymphocytes (PBL) stimulated with anti-CD3 antibody¹⁹. The amplified human V β region was joined in-frame to the expression vector, pBDWMC β 2DODJ. This new chimaeric TCR β -chain (human V-region, mouse DJ region and C chain) was transfected into the recipient mouse T-cell hybridoma, DS23-27.4, which expresses all the functional components of the TCR complex except the TCR β -chain (D.D. and E. Palmer, manuscript in preparation). The transfectants were selected by G418 resistance. The expression of human-mouse chimaeric TCR β -chain was screened by staining with either anti-CD3 (145-2C11) or anti- $\alpha\beta$ TCR (H57-597) antibody^{25,26}.

METHODS. Total RNA and cDNA were prepared as described previously¹⁹. Primers (5' and 3') for the polymerase chain reaction (PCR)²⁷ carried EcoRI and NheI sites, respectively. Amplified hV β n fragments were subcloned into pTZ18R vector, sequenced and ligated into expression vector pBDWMC β 2DODJ. The plasmid pBDWMC β 2DODJ, derived from pSFFVSVneo (gift of Dr S. Fine), contained D β J β C β 2 cDNA sequences cloned from the mouse hybridoma D011.10 (ref. 28). The expression vector carrying the appropriate hV β n genes was transfected into recipient murine T cells (DS23-27.4) by electroporation²⁹. The recipient line, DS23-27.4, was derived from an antigen-specific mouse T-cell hybridoma (DS23.27) by irradiation. The DS23-27.4 had lost its rearranged TCR β -chain genes, as confirmed by Southern blot analysis. The transfected cells were selected with 700 μ g ml⁻¹ G418. The hV β 13.1 sequence was amplified with the primers 5'-GGGAATTCAAGATGGCCATCGGCTCCTGTGCTGTGC-3' and 5'-TAACTGCTAGCACAGAA-GTACACAGATGTC-3'. The hV β 13.2 sequence was amplified with 5'-GGGAATTCAAGATGGCCCTCGGCTCCTGTGCTGTGG-3' and 5'-GCTGCTAGCACAGAA-GTACACAGATGTT-3' oligonucleotides. The sequences of hV β 13.1 and hV β 13.2 obtained in this study are consistent with those described previously^{30,31}. The hV β 13.1D2 sequence was derived from hV β 13.1 and



hV β 13.2 by joining 5' of hV β 13.1 sequences to 3' of hV β 13.2 at amino-acid position 66 by PCR, using the joining primer, GAAATCTGTTTTTAATCTGGA-GACATTGTAGCC-3' (ref. 32). Abbreviations: LTR, long terminal repeat; SV40 Ori, simian virus 40 origin of replication.

TABLE 2 Toxin presentation is not MHC class II allele-specific

Antigen-presenting cells	T cells	None	Units ml ⁻¹ IL-2 produced in response to <i>S. aureus</i> toxins					SED
			SEA	SEB	SEC1	SEC2	SEC3	
B10·A(5R) (I-A ^b I-E ^b)*	hVβ13.1-1	—†	—	—	—	—	—	—
	hVβ13.2-1	—	—	—	—	567	389	—
	hVβ13.1D2-1	—	—	—	—	712	311	26
B10·BR (I-A ^k I-E ^k)	hVβ13.1-1	—	—	—	—	—	—	—
	hVβ13.2-1	—	—	—	—	138	99	—
	hVβ13.1D2-1	—	—	—	—	540	167	—
B10·D2 (I-A ^d I-E ^d)	hVβ13.1-1	—	—	—	—	—	—	—
	hVβ13.2-1	—	—	—	—	529	246	—
	hVβ13.1D2-1	—	—	—	—	1,610	730	—
B10·Q (I-A ^q)	hVβ13.1-1	—	—	—	—	—	—	—
	hVβ13.2-1	—	—	—	—	186	79	—
	hVβ13.1D2-1	—	41	—	—	3,013	1,729	—
B10·HTT (I-A ^s I-E ^s)	hVβ13.1-1	—	—	—	—	—	—	—
	hVβ13.2-1	—	45	—	—	363	418	22
	hVβ13.1D2-1	—	76	—	36	4,975	1,814	74

The three T-cell hybridomas described in Table 1 were tested for IL-2 production in response to toxins presented by spleen cells from different strains of mice. Spleen cells were prepared by treatment with anti-T serum, followed by guinea pig complement treatment and irradiation (1,000 rad). Background IL-2 production from the toxin-treated, T-cell-depleted spleen cells alone (10–60 U ml⁻¹) were subtracted from that obtained with the T-cell hybridomas present.

* Haplotype of class II molecules are indicated in parentheses.

† <20 U IL-2 ml⁻¹ indicated by —.

Peptide Antigen+MHC Binding Site?

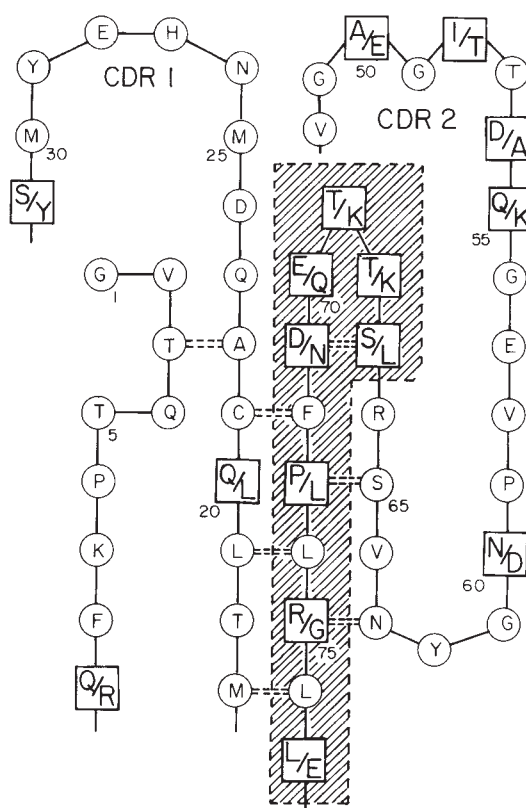


FIG. 2 Alignment of the amino acids (single-letter code) of hVβ13.1 and hVβ13.2 to the predicted structure of the TCR. The structure of a portion of Vβ shown here was adapted from a model of the TCR V domains by Chothia *et al.*²⁴ (see Fig. 3 in ref. 24). The amino acids differing in hVβ13.1 and hVβ13.2 are shown at their predicted positions as X/Y within squares, where X is amino-acid residue of hVβ13.1 and Y is hVβ13.2. The residues in common between hVβ13.1 and hVβ13.2 are shown in circles. The polypeptide hβ13.1D2 has the amino-acid residues of hVβ13.2 in the hatched box region and those of hVβ13.1 in the rest of TCR Vβ. The sites labelled CDR1 and CDR2 are predicted along with CDR3 (Dβ–Jβ, not shown) to form the portion of Vβ responsible for peptide antigen-MHC recognition.

T cells during thymic selection (P.M., M. Blackman, K. Choi and J.K., manuscript in preparation).

Finally, the human Vβ-expressing transfectants produced in these studies offer all the advantages of mouse T-cell hybridomas in that they are easy to maintain and produce in large quantities. The fact that all the components of TCR except Vβ are from mouse, renders this system suitable for the generation of mouse antibodies against particular human Vβ elements. In fact we have produced sero-positive animals by immunizing mice with these cell lines (Y.C., J. Lafferty, J. White, B. Kotzin, P.M. and J.K., manuscript in preparation). It should also be easy to modify this protocol, using an appropriate vector/recipient-cell system, to generate murine cells expressing human Vα elements. □

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The $\alpha 1$ domain of the HLA-DR molecule is essential for high-affinity binding of the toxic shock syndrome toxin-1

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SEVERAL exoproteins from the bacterium *Staphylococcus aureus* are highly potent polyclonal activators of T cells in the presence of cells bearing class II antigens of the major histocompatibility complex (MHC)¹⁻³. These toxins, including the toxic shock syndrome toxin (TSST-1), act at nanomolar concentrations, bind directly to class II molecules, and do not require the processing typical of nominal antigen³⁻⁷. Each toxin is capable of stimulating a subpopulation of peripheral T lymphocytes bearing particular V β sequences as part of their $\alpha\beta$ T-cell receptors^{8,9}. It is not known how these so-called 'superantigens' bind to class II and how this binding stimulates T cells. In this study, the different affinities of TSST-1 for human class II molecules DR and DP were exploited to define the region of a class II molecule necessary for high-affinity binding. Using chimaeric α - and β -chains of DR and DP expressed at the surface of transfected murine fibroblasts and a binding assay with TSST-1, it was shown that the $\alpha 1$ domain of DR is essential for high-affinity binding, and further that TSST-1 binding did not prevent subsequent binding of a DR-restricted antigenic peptide. This is compatible with a model of

superantigen making external contacts with both class II and T cell receptor, and suggests that the V β portion of the T-cell receptor interacts with the nonpolymorphic α -chain of DR.

Functional assays for T-cell activation reveal that all three expressed isotypes of human class II, HLA-DR, -DQ, and -DP, are capable of interaction with staphylococcal toxins^{5,10}. Several groups have demonstrated the direct, high affinity (dissociation constant $K_d \approx 10^{-9}$ M) binding of toxins to class II molecules^{4,6,7,11}. All isotypes and allotypes of human class II tested so far interact with at least one of the staphylococcal toxins, but there are several differences. For example, [¹²⁵I] TSST-1 binding to L cells transfected with HLA-DPw2 or -DPw4 cannot be demonstrated in a radio-ligand binding assay that easily detects binding to HLA-DR and -DQ alleles¹⁰. By contrast, T-cell stimulation by TSST-1 in the presence of DP-positive L cells was comparable to that seen with the other isotypes. This disparity was felt to represent the greater sensitivity of the cellular assay compared with direct binding assays.

L cells expressing either the DR or DP α -chain were transiently transfected with complementary DNAs for chimaeric β -chains¹². The cells were simultaneously tested for the ability to bind the staphylococcal toxin, TSST-1, and for class II expression. The TSST-1 binding assay has been shown to be dependent on the presence of class II molecules on the surface of the L cells and is completely inhibitable by the presence of excess unlabelled TSST-1 (ref. 6). This assay detects binding to various alleles of HLA-DR in stably transfected L cells over a wide range of levels of class II expression¹⁰. In addition to the wild-type DR β -chain, three chimaeric β -chains were each expressed in conjunction with the DR α -chain (Fig. 1)¹². The chimaeras RP22 and RP97 have DR β -chain sequence for the first 22 and 97 amino acids, respectively, and DP β -chain sequence for the remainder of the molecule. The chimaera PR43 has DP β -chain sequence for the first 43 amino acids and DR sequence for the rest of the β -chain. Taken together, the portion of the β -chain sequence derived from DP spans the entire β -chain. Cells bearing class II molecules consisting of any of

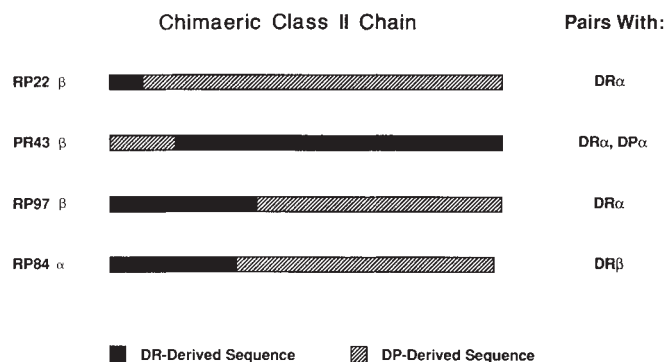


FIG. 1 Schematic diagram of chimaeric class II α - and β -chains. Chimaeric cDNAs having sequences derived from both DR (solid) and DP (hatched) α - or β -chains were constructed for subcloning into a polyoma-based eukaryotic expression vector. The ability of each chimaeric chain to pair, and be expressed with wild-type chains from either isotype is indicated. METHODS. The hybrid DR-DP β -chain constructs characterized previously were also used in this study¹². A chimaeric cDNA containing sequences derived from the $\alpha 1$ domain of DR and the $\alpha 2$ domain of DP was constructed using the technique of 'synthesis by overlap extension', and will be described in detail elsewhere (D.R.K., manuscript in preparation). Murine fibroblasts (L cells) that stably express either the DR or DP α -chain or the DR β -chain were transfected with the replicating vector having a wild-type or chimaeric partner chain cDNA, using DEAE-dextran to facilitate DNA uptake as described. The cells were cultured in 10 mM sodium butyrate for 24 h before analysis to maximize transfected class II gene expression. After transfection (48 h), the cells were analysed for ability to bind iodinated TSST-1 (see Fig. 2 legend). A portion of the cells was stained with the monoclonal antibody SG465 (a gift of Dr S. Goyert)²⁰ and analysed by microcytofluorimetry for class II expression.

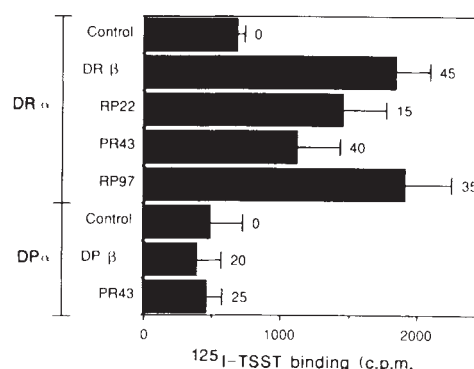
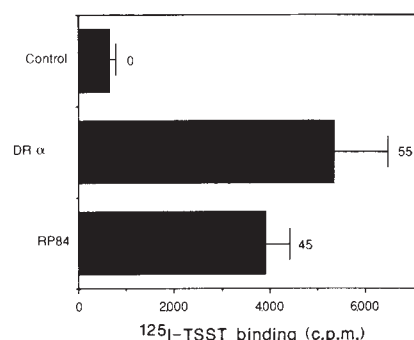


FIG. 2 Binding of iodinated TSST-1 to L cells expressing class II molecules having wild-type or chimaeric β -chains. Cells expressing either the DR or DP α -chains were transfected with either the wild-type isotypic β -chain or the indicated chimaeras. After transfection (48 h), 10^5 live cells were tested for their ability to bind [¹²⁵I]TSST-1. Controls for each α -chain were transfections with the β -chain of the non-homologous isotype, which is expressed intracellularly, but does not pair with the α -chain and does not appear at the cell surface. The mean and standard errors of triplicate determinations from one representative experiment are shown. The percentage of cells in each transfection that react with an anti-class II monoclonal antibody (SG465) is given to the right of each bar.

METHOD. Toxin-binding was assessed by incubating 10^5 live cells in the presence of 10^{-8} M [¹²⁵I]TSST-1 for 1.5 h on ice⁶. The cells were layered over a cushion of mixed phthalate oils and centrifuged in a 400 μ l polypropylene tube. The tips of the tubes containing the cell pellets were then placed in a gamma counter. The values from triplicate determinations were averaged.

FIG. 3 Binding of iodinated TSST-1 to L cells expressing class II molecules with a chimaeric α -chain. L cells that stably express the DR1 β -chain were transfected with cDNAs for the wild-type or PR84 α -chain and analysed for their ability to bind TSST-1 as above. Control cells were transfected with the DP α -chain, which is expressed intracellularly but does not form $\alpha\beta$ dimers at the cell surface (C.L.T., unpublished data). The percentage of SG465-positive cells is given to the right of each bar.



these chimaeric β -chains paired with DR α are capable of binding TSST-1 (Fig. 2). The percentage of cells stained by the anti-class II antibody, SG465, varies from 15–45%. The amount of toxin bound per 10^5 cells varied from chimaera to chimaera, but was reproducible in separate experiments, and not absolutely related to the level of class II expression. Therefore, the sequences necessary for the high affinity (DR-like) binding of toxin are not located in the β -chain, although the β -chain may exert an effect on the relative efficiency of this interaction.

One of these chimaeric β -chains (PR43) is also expressed with the DP α -chain. TSST-1 did not bind to cells transfected with the DP α -chain and PR43, although high levels of class II were expressed at the cell surface. As expected, cells transiently expressing the wild-type DPw2 molecule did not bind the toxin. Another β -chain chimaera, PR59, which is expressed with both DR and DP α -chains¹² was also tested for TSST-1 binding (not shown). As with the PR43 β -chain, toxin binding was only observed when PR59 was paired with the DR α -chain, and not when it was paired with the DP α -chain. This confirms the finding that it is the α -chain of DR that is required for the high affinity binding to TSST-1.

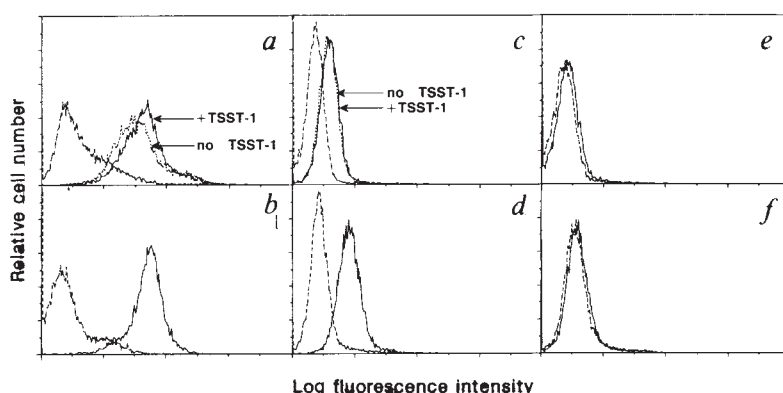
The cDNA coding for an α -chain having an $\alpha 1$ domain derived from DR and an $\alpha 2$ domain derived from DP was subcloned into the polyoma-based expression vector, R3CPy-II and transfected into murine L-cells that were stably expressing the DR β -chain. This chimaeric α -chain, RP84, pairs very efficiently with DR β and is expressed at high levels at the cell surface, but is not expressed at the cell surface with the DP β -chain (data not shown). Both the cells expressing the wild-type DR1 molecules and those expressing the chimaeric α -chain bind

TSST-1 (Fig. 3). This observation, along with the data presented above, demonstrate that the interaction of TSST-1 with HLA-DR requires sequences present in the $\alpha 1$ or amino-terminal portion of the α -chain. These data do not prove that the toxin is binding directly to $\alpha 1$ sequences, but that is the most straightforward interpretation. Binding may involve a combination of α - and β -chain sequences, or may even be to the β -chain alone, but strictly requires the presence of the DR α -chain for proper conformation. Repeated attempts to demonstrate binding of TSST-1 to α - or β -chains isolated by SDS-PAGE have been unsuccessful, suggesting that the toxin-binding epitope has either a conformational or combinatorial requirement.

It is not known whether the toxin stimulates T cells by inducing a conformational change in class II that is then recognized by the T-cell receptor (TCR), or alternatively by simultaneously contacting both class II and TCR. As either intact TSST-1 (relative molecular mass 22,000; M_r 22K) or a 14K fragment are required for functional activity^{13,14}, it is unlikely to bind within the postulated peptide-binding 'groove' formed by the $\alpha 1$ and $\beta 1$ domains of class II¹⁵, although this possibility exists. Additionally, TSST-1 may block access to the class II site by allosteric effects. To investigate this, class II-bearing cells were incubated in the presence of a biotinylated analogue of the main DR1-restricted antigenic peptide from influenza haemagglutinin [LCB-HA(307–319)] (ref. 16) with or without a prior incubation in saturating amounts of TSST-1. The cells were then stained with fluorescein isothiocyanate-conjugated avidin and rabbit anti-TSST (plus phycoerythrin labelled goat anti-rabbit immunoglobulin) and analysed by microcytofluorimetry. The B-cell line 45.1 expresses the HLA-DR1, -DQw1 and DPw2

FIG. 4 Binding of an antigenic peptide is unaffected by TSST-1. The B-cell line 45.1 and L-cell transfectants expressing DR1 and DPw2 were incubated in the presence of the synthetic peptide LCB-HA(307–319), with or without prior incubation with TSST-1. The cells were then stained as described below and analysed by microcytofluorimetry. a, c, Control staining (—); binding of LCB-HA(307–319) alone (· · ·); binding of LCB-HA(307–319) after binding of TSST-1 (—) to 45.1 (a) and DAP-DR1 (c). e, Control (—); binding of TSST-1 (—) to 45.1 (b), DAP-DR1 (d), and DAP-DPw2 (f).

METHODS. The peptide LCB-HA(307–319) was obtained from Dr J. Rothbard, (ImmuLogic Pharmaceutical Corp., Palo Alto, California). It has the sequence (N- to C-terminal): PRYVRQNTLRRLAT (single-letter amino-acid code) which corresponds to the substitution of arginine for lysine at positions 308, 311, and 316 in the native haemagglutinin sequence to prevent biotinylation of ϵ amino groups. The peptide was coupled to NHS-LC-biotin (Pierce) through the α -amino group. This peptide competes effectively for the native HA(307–319) in both direct binding and antigen presentation assays¹⁶. The hemizygous B-cell line, 45.1, has the HLA-D region haplotype DR1, DPw2, DQw1 (ref. 17). DAP-DR1 and DPw2 are clones of L-cell stably transfected with cDNAs for the α - and β -chains of DR1 and DPw2, respectively. Cells were incubated 5×10^5 for 1 h at 4 °C, in either 100 μl of complete medium or 100 μl of a $10 \mu\text{g ml}^{-1}$ solution of TSST-1 ($\sim 4 \times 10^{-7}$ M) diluted in complete medium. The cells were then diluted with either 100 μl of complete medium or a 200 mM solution of



LCB-HA(307–319). The cells were incubated at 37 °C for 6 h, then washed and stained as follows: FITC-avidin D (1:100, Vector Laboratories), wash, biotinylated goat anti-avidin (1:100, Vector Laboratories) plus rabbit anti-TSST-1 (1:100, Toxin Technologies, Madison, Wisconsin), wash, FITC-avidin again, plus phycoerythrin-labelled goat anti-rabbit Ig (1:100, Southern Biotechnology Associates, Birmingham, Alabama). After a final wash, the cells were analysed by two-colour microcytofluorimetry on the FACScan (Becton-Dickinson, Mountain View, California).

molecules on its surface¹⁷. These cells bound equivalent amounts of the peptide regardless of the presence of TSST-1 (Fig. 4a). That TSST-1 was actually bound to these cells is demonstrated in Fig. 4b. The data are shown as separate fluorescence histograms for clarity; two-colour fluorescence contour plots revealed a single homogeneous population of cells positive for both peptide and TSST-1 binding. Figure 4c demonstrates similar findings for a stable L-cell transfectant expressing DR1. The quantity of class II on these cells is about one-tenth that of the B cells. The level of peptide binding was correspondingly lower. Again, pre-incubation of the cells with TSST-1 did not affect the level of peptide binding. The binding of TSST-1 to these cells is shown in Fig. 4d. An L-cell transfectant expressing equivalent amounts of DPw2 did not bind either the biotinylated peptide or TSST-1 (Fig. 4e, f).

It has been estimated that ~1% of the surface DR molecules will bind the haemagglutinin peptide under the conditions used¹⁶. Presumably, the toxin is unable to distinguish those class II molecules that will eventually bind the biotinylated peptide from those that do not. The simultaneous binding of TSST-1 and peptide to the same DR molecule is then most consistent with the model of toxin contacting both the class II and the TCR outside of the peptide-binding site.

There is some evidence for a functional interaction between the α -chain of class II and the β -chain of the T cell receptor. The maximal response of murine T cells to a mitogen from *Mycoplasma arthritidis*, which is predominantly by cells bearing V β 6 and V β 8 TCRs, requires the presence of the I-E α -chain¹⁸. L cells expressing either the E α -E β or E α -A β murine class II molecules were far better stimulators of purified T cells in the presence of mycoplasmal mitogen than were fibroblasts expressing A α -A β . Also, the expression of an E α transgene in H-2^b mice that do not normally express E α leads to the ability of the mitogen to stimulate splenocytes *in vitro*. This is consistent with the hypothesis that this mitogen requires the presence of E α for binding to class II. In addition, Berg, *et al.*¹⁹ have studied the selection of the T-cell repertoire in TCR transgenic mice. Mice that express transgenes for particular TCR α - and β -chains have strict requirements for the class II haplotypes that will lead to positive selection of the transgenic TCR. However, mice that express the transgenic TCR β -chain in association with a variety of endogenous TCR α -chains are selected by several I-E haplotypes (all of which share a nonpolymorphic class II α -chain), suggesting that the TCR V β region interacts with the class II α -chain. These data argue for a functional, if not physical, interaction between the α -chain of I-E and the V β region of the TCR. The finding that the high-affinity binding of TSST-1 to HLA-DR requires the α 1 domain is consistent with this hypothesis, and suggests that there are contact points between the DR α -chain and the V β domain of the TCR. □

Empty MHC class I molecules come out in the cold

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MAJOR histocompatibility complex (MHC) class I molecules present antigen by transporting peptides from intracellularly degraded proteins to the cell surface for scrutiny by cytotoxic T cells. Recent work suggests that peptide binding may be required for efficient assembly and intracellular transport of MHC class I molecules¹, but it is not clear whether class I molecules can ever assemble in the absence of peptide. We report here that culture of the murine lymphoma mutant cell line RMA-S at reduced temperature (19–33 °C) promotes assembly, and results in a high level of cell surface expression of H-2/β₂-microglobulin complexes that do not present endogenous antigens, and are labile at 37 °C. They can be stabilized at 37 °C by exposure to specific peptides known to interact with H-2K^b or D^b. Our findings suggest that, in the absence of peptides, class I molecules can assemble but are unstable at body temperature. The induction of such molecules at reduced temperature opens new ways to analyse the nature of MHC class I peptide interactions at the cell surface.

A mouse mutant lymphoma cell line, RMA-S, expresses at the cell surface <10% of the amount of class I molecules compared with RMA mutagenized but unselected control line^{2,3}—and is unable to present endogenous antigens^{1,4}. Rates of synthesis of class I heavy and light chains are normal in RMA-S, and analysis of RMA-S-L-cell hybrids excludes the possibility that the defect involves a mutation in the H-2 heavy or light chain of RMA-S⁵.

In an experiment originally designed to examine the contribution of the various protein breakdown routes⁶ to reduced surface expression in RMA-S, cells were pulse-labelled at 37 °C and chased at 26 °C. At this temperature, essentially no breakdown of class I molecules of the RMA wild-type line occurred, and glycan modifications were slower compared with chase at 37 °C (Fig. 1a). Breakdown of class I molecules was also decreased at 26 °C for RMA-S but, surprisingly, extensive modifications of N-linked glycans were observed (Fig. 1a).

Culture of RMA-S at temperatures ranging from 8–35 °C (optimum 23–31 °C) also led to a marked increase in the expression of K^b and D^b at the cell surface (Fig. 2a). Expression increased linearly with time (shown for 22 °C), up to 28 h (Fig. 2b). Culture of RMA-S at 26 °C for 24 h (notation, RMA-S (26 °C)) was adopted as the standard treatment, unless stated otherwise. The class I molecules that appear at the cell surface at low temperature seem to be folded correctly, and are associated with β₂-microglobulin. For both D^b and K^b, a parallel increase in the binding of monoclonal antibodies to epitopes on the α1, α2 or α3 domains of the heavy chain, as well as to β₂-microglobulin was observed (Fig. 2c). Comparison of surface-labelled material of RMA-S (26 °C) and RMA-S (37 °C) immunoprecipitated with either anti-H-2^b serum or the confor-

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mation-independent anti-K^b exon 8 antibody (a gift from Dr B. Barber) reveals the existence of class I molecules on RMA-S (37 °C) that are not recognized by the anti-H-2^b serum (possibly reflecting the population of H-2 molecules that can be stabilized by addition of peptide at 37 °C). But there is a significant increase in the amount of material recovered with both antisera on culture at reduced temperature (data not shown). The effect of low temperature on class I expression on RMA-S differs from the effect of peptides added at 37 °C¹. Low temperature increases the expression of both D^b and K^b, whereas peptides have predominantly allele-specific effects¹. The two effects are additive during the first 6 h of incubation at 26 °C (data not shown). The effect of low temperature on class I expression was also observed, but to a lesser extent, on RMA under identical conditions (Fig. 2b).

RMA-S has been reported to be incapable of presenting internally derived antigens to H-2-restricted cytotoxic T lymphocytes (CTL) specific for viral¹, minor histocompatibility or tumour antigens⁴. RMA-S (26 °C) remained resistant to H-2^b-

restricted CTL directed against minor histocompatibility antigens (Fig. 3a), despite a 5- to 10-fold increase in H-2K^b and D^b expression at the cell surface measured in parallel by cytofluorimetry (data not shown). Similarly, the RMA-S (26 °C) cells remained resistant to a H-2D^b-restricted influenza-specific CTL clone after virus infection at 26 °C (Fig. 3b). But when tested with a H-2D^b-restricted peptide, NP 366-379¹, RMA-S (26 °C) was 100-fold more susceptible to lysis than RMA-S (37 °C) in terms of the peptide concentration required to induce 50% killing (Fig. 3c). Although RMA-S (26 °C) had half the H-2D^b expression of RMA (37 °C), RMA-S (26 °C) could be sensitized at a far lower concentration of peptide than RMA (37 °C). Note that the peptide titration curve shifted also for RMA (26 °C).

We propose that the class I molecules exported to the cell surface at 26 °C in RMA-S lack peptide in their antigen-binding pocket. Our previous experiments suggested that peptide is required for assembly and stabilization of class I molecules at 37 °C in RMA-S¹. To test whether the class I molecules induced in RMA-S (26 °C) were unstable at physiological temperature,

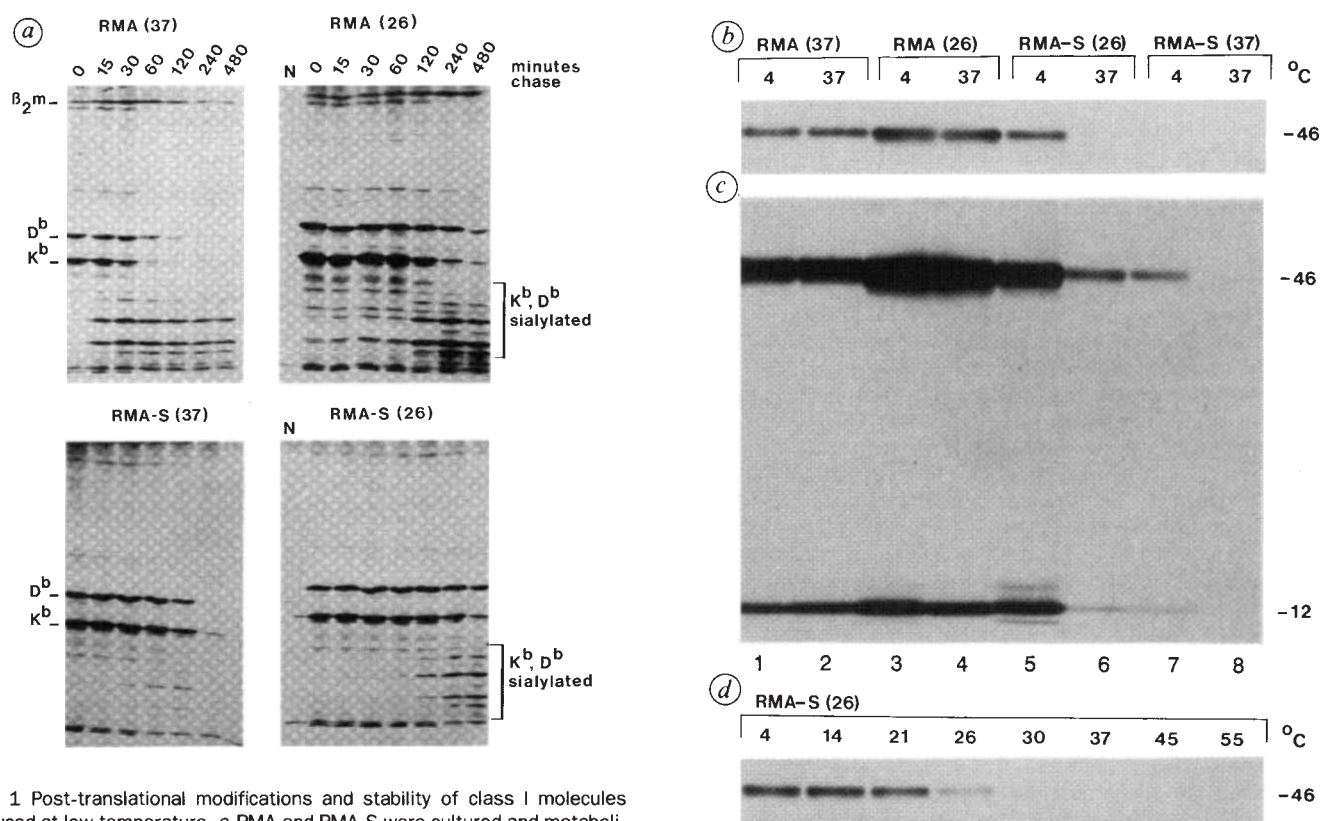


FIG. 1 Post-translational modifications and stability of class I molecules induced at low temperature. **a**, RMA and RMA-S were cultured and metabolically labelled for 10 min at 37 °C, chased for the times indicated at either 26 °C or 37 °C, and lysed. Class I molecules were immunoprecipitated and analysed on 1D-IEF gels. The position of β_2m -microglobulin (β_2m) and nonsialylated H-2K^b and D^b are indicated on the left, sialylated forms of K^b and D^b by the brackets on the right. Class I molecules showed a significantly prolonged half life at 26 °C in comparison with 37 °C for both RMA and RMA-S. Importantly, the extent of terminal glycan modifications (sialylation), indicating transport through the trans-Golgi, was higher in RMA-S chased at 26 °C than at 37 °C. **b**, **c**, RMA and RMA-S were cultured at either 37 °C or 26 °C, surface-labelled by iodination and lysed. Lysates were incubated at the temperatures indicated. Class I molecules were then immunoprecipitated and analysed by SDS-PAGE. Note the disappearance of anti-H-2 reactive material of RMA-S (26 °C) on incubation of the lysate at 37 °C (only the heavy chain region of the gel is shown in **b**). The small amounts of H-2 that are expressed on the surface of RMA-S (37 °C) exhibit similar thermolability (**c** is a longer exposure of **b**). **d**, Incubation of RMA-S (26 °C) lysates was performed at different temperatures, as indicated. Note the reduction in immunoreactive H-2 class I molecules after incubation at temperatures higher than 26 °C.

METHODS. **a**, Ten million cells were pulse-labelled with 500 μ Ci [³⁵S]methio-

nine (1,200 Ci mol⁻¹, Amersham) for 10 min at 37 °C and chased in the presence of excess of cold methionine for different periods at either 26 °C or 37 °C (ref. 14). After lysis, free β_2m was removed by immunoprecipitation using an anti- β_2m serum (K297, ref. 15). Specific immunoprecipitation used a rabbit anti-mouse H-2 serum (a gift from L. Rask, Uppsala; ref. 16 and S. Nathenson, New York) and analysed on 1D-IEF as described (ref. 17 and H.G.L., M. Oudshoorn-Snoek, M. G. Masucci and H.L.P., manuscript submitted). The anode is at the bottom. **b**–**d**, Cells were cultured for 48 h at 26 °C or 37 °C in RPMI-1440 medium, fetal calf serum (FCS). Five million cells were surface-labelled by lactoperoxidase-catalysed iodination as described¹⁸. Cells were lysed on ice in 1 ml lysis buffer (1% Triton X-100, 140 mM NaCl, 10 mM Tris buffer, pH 7.8, 1 mM phenylmethylsulphonyl fluoride, 1 μ g ml⁻¹ trypsin inhibitor, 1 μ g ml⁻¹ leupeptin, 30 mU of trypsin inhibitor ml⁻¹ aprotinin (Sigma)). The lysates were incubated at the temperature indicated for 1 h and transferred to 0 °C. H-2 class I were immunoprecipitated with rabbit anti-H-2 serum (see above) as described¹⁷, and analysed by SDS-PAGE on a 12% gel. Relative molecular mass standards ($M_r \times 10^{-3}$) are to the right.

H-2 expression was examined after shifting the cells to 37 °C. Brefeldin A (BFA) was added to prevent further transport of class I molecules from the endoplasmic reticulum to the cell surface^{7,8}. Within 1 h, more than half the D^b class I molecules induced at 26 °C had disappeared from the cell surface (Fig. 4a). After 4 h at 37 °C only trace amounts of induced K^b and D^b molecules remained (Fig. 4b). A similar decrease was observed for K^b and D^b in BFA-treated RMA-S (37 °C) cells (Fig. 4a, 4b), in agreement with the rapid decay of biosynthetically labelled class I molecules in RMA-S (37 °C). By contrast, more than 80% of class I molecules was still detectable at the surface after 2 h, in BFA-treated RMA (37 °C) cells and their half-life was >6 h as measured by cytofluorimetry (data not shown).

Significant amounts of surface-iodinated H-2 class I molecules, comparable to the amounts seen in RMA (37 °C), could be immunoprecipitated from RMA-S (26 °C) (compare lanes 1 and 5, Fig. 1b, c). When lysates were incubated at 37 °C, a complete loss of immunoreactive class I molecules of RMA-S (26 °C) or (37 °C) was observed (compare lanes 5 and 6; Fig. 1b-d). For RMA-S lysates, but not for RMA lysates, there was a sharp drop in quantity of H-2 recovered, with increasing temperature (Fig. 1d). The loss of H-2 class I molecules in the lysates from RMA-S was not due to proteolysis, as judged by the continued presence of H-2K^b and D^b heavy chains revealed by two-dimensional isoelectric focusing (IEF)/SDS-PAGE analysis of the total lysate (data not shown).

If the class I complexes induced at the RMA-S cell surface at low temperature are unstable at 37 °C because they are devoid of peptides, addition of class I-binding peptides to BFA-treated cells before transfer to 37 °C, should stabilize them. In accordance with their known restriction specification, this was indeed the observed result for epitopes presented in the context of H-2K^b and D^b (Fig. 4b, c), at concentrations of peptide, previously shown to restore H-2 expression in RMA-S (37 °C). This peptide-mediated stabilization could be confirmed by biochemical analysis of surface-labelled RMA-S (26 °C)⁹.

In summary, culture of RMA-S at low temperature induces MHC class I molecules at the cell surface that: (1) undergo post-translational modifications characteristic of passage through the trans-Golgi (Fig. 1a); (2) are associated with β_2 -microglobulin at the cell surface and express conformational epitopes found in properly folded class I molecules (Figs 1c and 2); (3) are unstable at 37 °C (Figs 1b-d and 4a-b); (4) fail to present internally derived antigens to CTL (Fig. 3a, b); (5) present exogenous peptides more efficiently than class I molecules of the wild-type line cultured at 37 °C (Fig. 3c), and (6) are stabilized by exogenous peptides in an allele-specific manner (Fig. 4b, c). The most simple interpretation is that MHC class I molecules devoid of internally derived peptides can reach the cell surface at the reduced temperature.

We propose the following explanation for our findings. Under normal conditions, the MHC class I subunits will combine with peptide early in biosynthesis. If they fail to do so, a heterodimer may fail to form or, if devoid of peptide, have a high probability of dissociating or displaying a grossly altered conformation at 37 °C. We surmise that such 'empty', aberrantly folded heavy chains would not be transported further and be rerouted or degraded. That fraction of class I/ β_2 -microglobulin heterodimers that reaches the cell surface empty at 37 °C can be stabilized by a suitable peptide supplied during experimental procedures. The intrinsic instability of empty class I molecules can be overcome not only by addition of peptide, but also by reducing the temperature. The affinity of the class I heavy chain for β_2 -microglobulin is thus sufficiently high at the reduced temperature to allow an increase in net transport of the complex to the cell surface in the absence of peptide.

The findings reported here underscore the role for peptide in maintaining structural integrity of class I molecules, but are consistent with the possibility that much of the increase in H-2

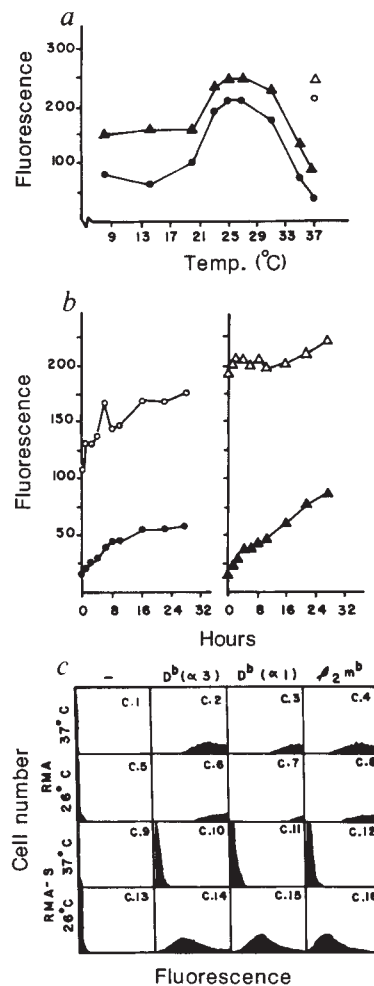
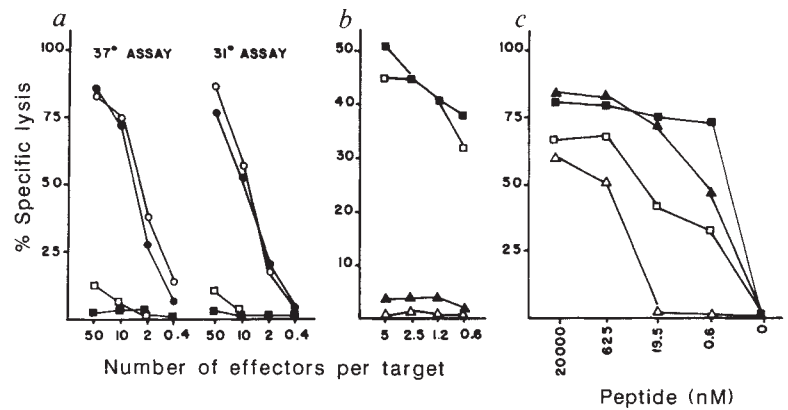


FIG. 2 Induction of cell surface class I molecules at low temperature. *a*, Staining with monoclonal antibodies against K^b (28-13-3S; ref. 19) and D^b (28-14-8S; ref. 20-22) at the cell surface of RMA-S cells cultured for 24 h at different temperatures. Quantitation of K^b and D^b expression on the control line RMA cultured at 37 °C is indicated for comparison. ●, RMA-S anti-K^b; ▲, RMA-S anti-D^b; ○, RMA anti-K^b; △, RMA anti-D^b. On average, in a series of 10 experiments, K^b and D^b expression increased to a level corresponding to 40–55% of RMA (37 °C). In occasional experiments the level of expression reached that of RMA (37 °C). *b*, Kinetics of class I increase at the cell surface measured with monoclonal antibodies against H-2K^b (28-13-3S) and D^b (28-14-8S) at 22 °C on RMA-S and RMA. ●, RMA-S anti-K^b; ▲, RMA-S anti-D^b; ○, RMA anti-K^b; △, RMA anti-D^b. *c*, H-2D^b induced at low temperature is associated with β_2 m and expresses a conformation-dependent epitope on the α 1 domain. Cell surface staining with monoclonal antibodies 22-14-8S (D^b, α 3; charts C.2, C.6, C.10 and C.14), B22.249 (D^b, α 1; charts C.3, C.7, C.11, C.15; ref. 21–23) and mc- β_2 m-B (β_2 m allele; charts C.4, C.8, C.12, C.14) of RMA (charts C.1–C.8) and RMA-S (charts C.9–C.14) cultured for 24 h at 26 °C or continuously at 37 °C. Charts C.1, C.5, C.9 and C.13 show background staining with no first (specific) antibody (–). Similar results were obtained for K^b with the antibodies Y3 (α 2, ref. 25) and S19.8 (ref. 26) (data not shown).

METHODS. All cells were cultured in RPMI-1440, 10% FCS supplemented with antibiotics. Cells were removed from 37 °C (5% CO₂), cultured (10⁶ cells ml⁻¹) in tissue-culture flasks (Falcon, 50 ml) with tightened cap, placed in a heat-adjustable water bath for 24 h at a given temperature unless noted otherwise. Culture at temperatures <8 °C for 24 h or more, usually reduced viability of cells. For cytofluorimetry, 10⁶ cells were incubated with 0.1 ml anti-class I monoclonal antibody tissue culture supernatant for 30 min on ice, washed twice in PBS, and then incubated with 0.1 ml fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin (Z109; DAKO, Copenhagen, Denmark) on ice for 30 min, washed twice in PBS, fixed in 1% formaldehyde and analysed on a FACS IV cell sorter. Results from *a* and *b* derived from mean linear fluorescence values obtained by FACS analysis. Monoclonal antibodies 28-14-8S and 28-13-3S were obtained from ATCC (Rockville, MD), B22.249 was a gift from G. J. Hammerling, mc- β_2 m-B (ref. 27) was a gift from E. A. Boyse.

FIG. 3 Sensitivity to cytotoxic effector cells after induction of class I molecules at low temperature on RMA and RMA-S. **a**, RMA-S (26 °C) cells are not recognized by CTL specific for minor histocompatibility antigens in ^{51}Cr release assay at 37 °C (left chart) or at 31 °C (right chart). Target cells were: ●, RMA (37 °C); ○, RMA (26 °C); ■, RMA-S (37 °C); □, RMA-S (26 °C). Note that this resistance was also observed if the cytotoxicity assay was at 31 °C (Fig. 3a), that is, within the temperature range permissive for high levels of cell surface expression of class I molecules (Fig. 2a). **b**, Low temperature treated RMA-S cells infected with a recombinant vaccinia that expresses a rapidly degraded C-terminal fragment of influenza NP (IMP-VAC, ref. 28) are also not recognized by the NP-specific CTL clone, F5. Target cells were: □, RMA (37 °C) infected with IMP-VAC; ■, RMA (26 °C) infected with IMP-VAC; △, RMA-S (37 °C) infected with IMP-VAC; ▲, RMA-S (26 °C) infected with IMP-VAC. Uninfected cells were not lysed above 1% after treatment at either temperature (not shown). **c**, Recognition by clone F5 of RMA and RMA-S after incubation at 26 °C or 37 °C and exposure to decreasing concentrations of peptide NP 366–379 (ref. 1). △, RMA-S (37 °C); ▲, RMA-S (26 °C); □, RMA (37 °C); ■, RMA (26 °C). The CTL clone F5 was used at a killer/target ratio of 5/1 throughout. **METHODS**. CTL in **a** were generated by secondary mixed lymphocyte culture across a minor histocompatibility barrier (A.BY anti-B6) as described³. ^{51}Cr -labelling of targets were performed at 26 °C and 37 °C, respectively. A standard protocol for the ^{51}Cr release assay was used³. Clone F5 in **b** was isolated from an influenza virus-infected B6 mouse as described²⁴. In **b** it was tested against RMA and RMA-S infected with a recombinant vaccinia that expressed the C-terminal portion of influenza NP (amino acids 327–498) under the control of the vaccinia virus 7.5K promoter²⁸. Target cells were incubated at 26 °C or 37 °C for 24 h, collected and infected with 10 plaque-forming units per cell of recombinant vaccinia, and labelled with ^{51}Cr , at the



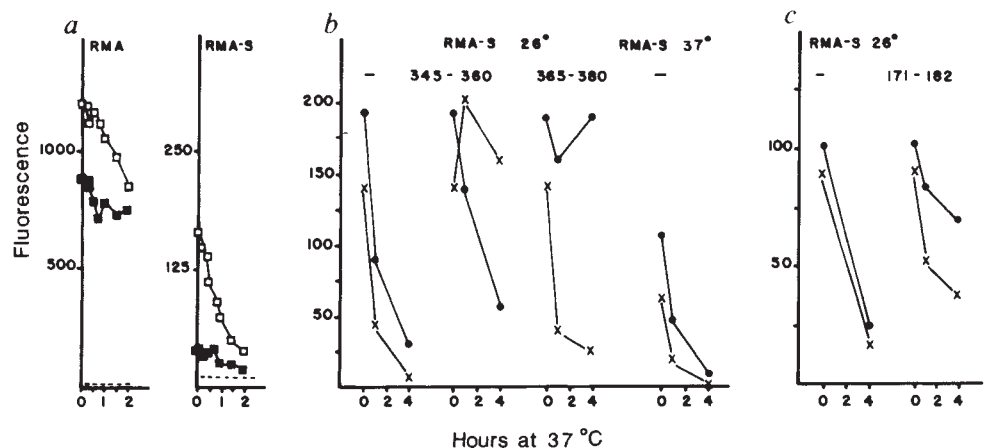
same temperature for 90 min. After washing, the cells were again resuspended at 26 °C or 37 °C for 4 h to allow for synthesis of vaccinia-encoded proteins. Cells were again washed and set up in a 4 h ^{51}Cr release assay as described at 37 °C (ref. 29) with CTL clone F5 at the killer/target cell (K/T) ratios shown. Surface expression of D^b measured with the monoclonal antibody B22.249 (mean fluorescence) measured after incubation at the different temperatures was 20, RMA-S (37 °C); 44, RMA-S (26 °C); 277, RMA (37 °C); 328, RMA (26 °C). In **c**, RMA-S and RMA were collected after 24 h at 26 °C or 37 °C, labelled with ^{51}Cr and incubated with peptide NP 366–379 (ref. 29) at the same temperatures at the concentrations shown for 90 min, before washing and exposing to CTL clone F5 at a K/T ratio of 5:1 for a 4 h ^{51}Cr -release assay at 37 °C. Surface expression of D^b measured with the monoclonal B22.249 after incubation at the different temperatures, but before addition of peptide, was; 9, RMA-S (37 °C); 44, RMA-S (26 °C); 238, RMA (37 °C); 318, RMA (26 °C).

surface expression that is induced by peptide¹, involves stabilization of empty molecules at the cell surface. Indeed, empty class I molecules can efficiently bind peptide in cell lysates^{9,10}. The quantity of peptide taken up by RMA-S through endocytosis is sufficient for stabilization of pulse-labelled H-2 molecules in such lysates¹⁰. There is therefore no evidence in favour of

retrograde transport of peptide to the ER where it would induce assembly.

Low-temperature induction of heterodimer assembly in the absence of peptide is a novel phenomenon that opens new ways not only to analyse peptide-MHC class I interactions, but also their role in various functions ascribed to MHC class I

FIG. 4 Class I molecules induced at low temperature on RMA-S are not stable at 37 °C but can be stabilized by peptides added exogenously. The influenza peptide NP 365–380, which is a defined epitope presented by D^b (ref. 29), prevented decay of D^b induced at 26 °C but not of K^b (Fig. 4b). The opposite pattern was seen for the peptide NP 345–360, which stabilized K^b but not D^b (Fig. 4b). The stabilization lasted for at least 4 h. It was readily observed at a peptide concentration of 10⁻⁴ M, but was not detectable at concentrations below 10⁻⁷ M (data not shown). These concentrations of peptide are similar to those required to restore class I cell surface expression in RMA-S at 37 °C (ref. 1). A H-2D^b-derived sequence (residues 171–182), which competes for H-2D^b-restricted presentation of peptide³⁰ could also stabilize D^b molecules on RMA-S (26 °C) (Fig. 4c). A partial but reproducible stabilization of K^b was also observed and is consistent with a low affinity of H-2K^b for the peptide¹. The decay curves for RMA preincubated at 26 °C and 37 °C converged, suggesting that preincubation at the lower temperature increased the proportion of unstable class I molecules. The data are consistent with the existence of two populations of class I molecules with respect to half-life. One induced at 26 °C on both RMA-S and RMA with a half-life of ~1 h at 37 °C, the other found only on RMA and stable at 37 °C with a half-life >6 h. **a**, Decay of H-2D^b on RMA (left chart) and RMA-S (right chart) precultured for 24 h at 26 °C (□) or at 37 °C (■), then incubated at 37 °C in the presence of brefeldin A. Cells were stained with monoclonal B22.249 (D^b, α1) and analysed by FACS (see Fig. 2), at different timepoints after transition to 37 °C. Background staining with



secondary fluorescent antibody alone indicated (—). **b**, Influenza nucleoprotein-derived peptides prevent decay of induced class I molecules on RMA-S (26 °C). Samples were stained and fixed for FACS analysis (see Fig. 2) after 0, 1, and 4 h at 37 °C, with monoclonals against K^b (×, 28-13-3S); and D^b (●, 28-14-8S). Stabilization generally lasted for at least 4 h. It was readily observed with peptide concentrations of 100 μM or more and was not detected at concentrations below 0.1 μM. **c**, Peptides derived from a conserved region of the H-2D^b molecule (residues 171–182) prevent decay of K^b less efficiently than decay of D^b. Experimental conditions as in **b**. **METHODS**. BFA (2 μl, 5 mg ml⁻¹ in methanol) was added to a mixture of 0.9 ml of cells in RPMI-FCS and 0.1 ml peptide NP(1934) 365–380 or NP (1968) 345–360, giving a final concentration of 10 μg ml⁻¹ BFA and 200 μM peptide. In controls, 0.1 ml medium was added without peptide. The mixture of cells, BFA and peptide was then immediately put at 37 °C (5% CO₂).

molecules. Apart from antigen presentation to class I-restricted CTL¹, these functions include interactions with other cell surface molecules¹¹ and target recognition by alloreactive CTL^{4,12} and natural killer cells^{2,3,13}. □

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Specific expression of a silk-encoding gene of *Bombyx* in the anterior salivary gland of *Drosophila*

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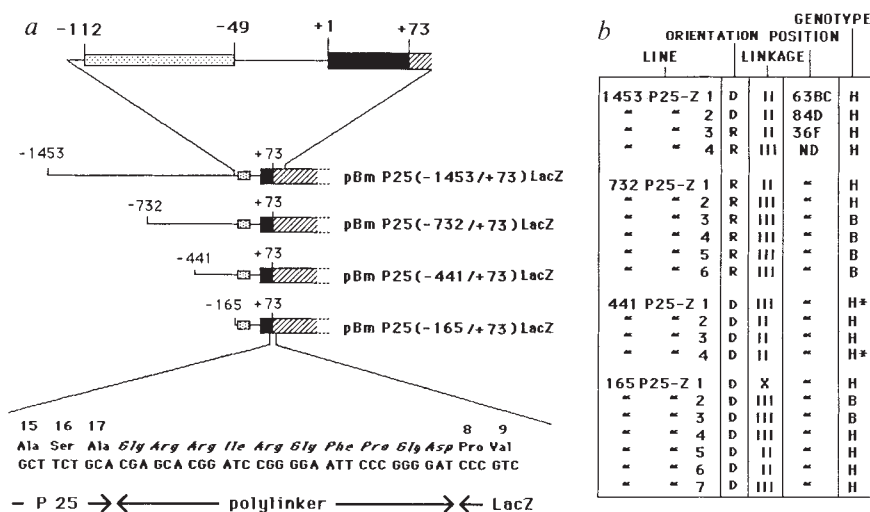
SUCCESSFUL expression of genes transferred into distantly related species in which genetic functions have been maintained through evolution has been reported previously¹⁻³. In the case of

the silkworm *Bombyx mori* and the fruitfly *Drosophila melanogaster*, both of which produce chorions (eggshells), *Bombyx* chorion genes are correctly expressed in *Drosophila* despite their estimated 240-Myr phylogenetic divergence¹. Here we report that, although *Drosophila* does not produce silk, mechanisms regulating transcription have been conserved between the salivary gland of the fruitfly and the silk gland of the silkworm larva.

The P25 gene of *Bombyx mori* is one representative of this set of silk protein genes expressed in the posterior silk gland⁴⁻⁷. Different fusion genes were made by joining the *Escherichia coli* β -galactosidase coding sequence to the P25 gene with either 1,453, 732, 441 or 165 base pairs (bp) of P25 5' flanking sequences. In all constructs, the fusion occurred at position +73 of the *Bombyx* gene, 54 bp downstream from the initiation codon (Fig. 1). These sequences were inserted into the vector carnegie 20, which carries the P-element terminal repeats and the gene *rosy* for phenotypic selection of transformants⁸. To account for a possible influence of the *Drosophila rosy* promoter on the

FIG. 1 P25-lacZ constructs used for *D. melanogaster* transformation experiments and the transformed lines analysed. *a*, Schematic representation of the chimaeric genes. P25 genomic fragments with various lengths of 5' upstream sequences (thin line) and the first exon (filled box) were fused in frame with the *E. coli* β -galactosidase coding sequence (hatched box). Numbers represent the distances in nucleotides from the transcription start site. Dotted box, the enhancer element of the P25 gene (J. Drevet, B. Durand and P.C., unpublished results). The fused genes were inserted into *Drosophila* chromosomes using P-element mediated transformation and *rosy* selection of transformants. *b*, The transformed lines were genetically mapped and made homozygous (H) for the insert, through crosses with balancer stocks. Lethal (B) or sterile (H*) insertions were maintained over appropriate balancer chromosomes. All the lines were shown to carry a single copy of the transgene by probing Southern blots with P25 and lacZ DNA. Localization of the insert was determined by *in situ* hybridization to polytene chromosomes with a P25-Z probe. Transcriptional orientation of the fused gene was either the same (D) or the opposite (R) to that of the *rosy* gene in the transformation vector injected.

METHODS. P-element vectors containing P25-lacZ fusion genes were constructed using standard cloning procedures. pBmP25(-1,453/+73)lacZ was constructed by sequential insertion in the plasmid pUC18 of the *HindIII*-*PstI* (-1,453/+73) fragment of the P25 gene and the *PstI*-*PstI* 3 kilobase (kb) fragment of the plasmid pMC1871 (ref. 12) containing the lacZ coding region with polylinker sequence. The same procedure was used to construct pBmP25(-732/+73)lacZ, with the *HincII*-*PstI*(-732/+73) fragment. The plasmids containing P25(-441/+73) and P25(-165/+73) sequences were



obtained by partial *Bal*31 digestion of pBmP25(-732/+73)DNA. End points of *Bal*31-digested DNA and the junction regions of P25 to lacZ sequences were ascertained by DNA sequencing. Fragments carrying P25-lacZ constructs were inserted into the *Hpa*I site of the carnegie 20 vector⁸. *Drosophila* transformation was as described⁹ using the p π 25.7wc helper DNA. The recipient strain (*ry*, *st*, *ecd*) was provided by J. A. Lepesant. Autosomal insertions were made homozygous by crosses with the *CyO*; *TM3ry*^k/T(2;3)Ap^{Xa} balancer stock. Lines that could not be made homozygous for the transgene were maintained as balanced stocks with *CyO* or *TM3* chromosomes. The X-linked insertion was made homozygous by inbreeding.

expression of the fused gene, their respective transcriptional orientation within the vector was made either the same or opposite (Fig. 1). The constructs were injected with helper DNA (p π 25.7 wc) (ref. 9) into *Drosophila rosy* and *scarlet* embryos. Transformant lines were made homozygous or were stabilized with appropriate balancer chromosomes in the case of lethal insertions. Four to seven independent lines were established per construct, each being characterized by a unique *Drosophila* genomic DNA insertion site (Fig. 1).

The expression of the transgene was assayed by histological detection of the bacterial β -galactosidase activity using X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) as chromogenic substrate on tissues of animals taken at different stages, from early first larval instar to pigmented puparium, and during early and late adult life. The staining pattern of each line was compared to that of the recipient strain and to that of other laboratory stocks.

In all the lines transformed with either the 1,453, the 732 or the 441 P25-Z fusion genes and whatever their orientation within the vector, expression was detected in the larval—but not in the adult—salivary glands, where no endogenous activity was found in controls. No other organs displayed detectable staining, except the midgut, where staining developed as in the control strains. To confirm that the salivary gland staining was dependent on the expression of the transgene, we detected the enzyme in this tissue on polyacrylamide gels. The active protein migrated with a relative molecular mass (M_r) of $\sim$ 520,000 (520K), a little slower than the pure homotetramer of the *E. coli* enzyme—consistent with the presence of 20 extra amino acids in the fusion protein—identifying it as the product of the transgene. As expected, the *Drosophila* endogenous enzyme migrated with an apparent M_r of 160K (ref. 10) (gel not shown).

It is of interest that the activity of the transgene is restricted to the anterior salivary glands of *Drosophila*, whatever the larval stage or the construct analysed (Fig. 2). The sharp transitions between the β -galactosidase-positive and -negative cells delineate a group of 16–24 cells in the most anterior part of the gland proper and immediately adjacent to the duct cells. To our knowledge, the functional specialization of this territory with a discrete number of cells had not been described before¹¹ and remarkably, it occurs at an early stage of larval life.



FIG. 2 Typical β -galactosidase activity in anterior salivary glands of transformed *Drosophila*. Pictures show X-Gal staining of whole glands from larvae of the line 1,453 P25-Z 3 taken at the first larval instar (a) and at mid-third larval instar (b). β -Galactosidase histochemical detection was on whole dissected animals¹³. Transgene-dependent blue staining was observed only in the larval anterior salivary glands in the lines transformed with constructs carrying 1,453, 732 or 441 bp of P25 5' flanking DNA. Colour developed within 30 min–4 h in these lines, whereas no staining was detected until at least 48 h in the lines carrying 165 bp of 5' flanking sequence or in the controls (not shown). SG, salivary gland; D, duct; FB, fat body lobe.

Lack of β -galactosidase expression in the 165-P25-Z transformants limits the *cis*-elements needed for expression in *Drosophila* anterior salivary glands cells to within –441/–165, a region upstream of the enhancer of the gene (Fig. 1).

To characterize the level of expression of the transgene more thoroughly, we quantified the β -galactosidase activity in the

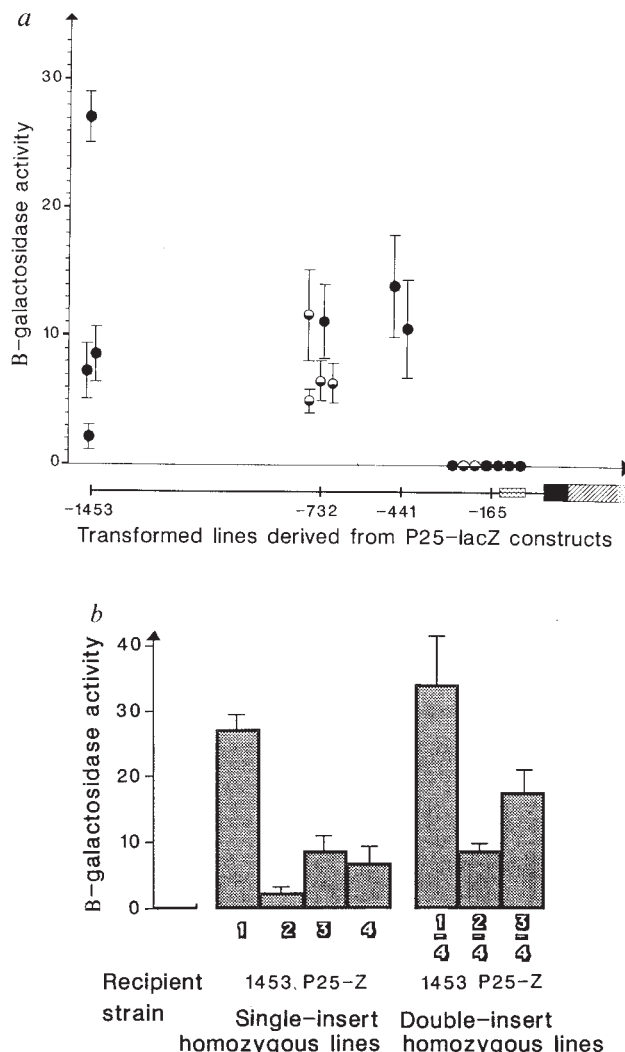


FIG. 3 Quantitative analysis of β -galactosidase activity in transformed strains carrying the P25-lacZ fusion gene and deletion mutants. a, Enzyme activity of salivary gland extracts prepared from wandering third instar larvae. For each transformed line, data were obtained from at least 3 independent sets of glands. The P25 flanking DNA is depicted at the bottom of the figure as in Fig. 1. Lines 441 P25-Z 1 and 2, having a very low fertility, were not included in the quantitative analysis but revealed expression of the transgene by histochemical tests. (●, Homozygous lines; ○, heterozygous balanced lines). b, Effects of gene dose on β -galactosidase activity. Double-insert lines carrying the 1,453 P25-Z fusion gene were obtained by crossing line 4 (insert on the third chromosome) to each of the lines 1, 2 and 3 (insert on the second chromosome). The double homozygous lines were assayed for β -galactosidase activity as in a, except that 8–13 independent assays were performed per line. β -galactosidase activity is given as absorbance₅₇₄ $\times 10^{-3}$ h⁻¹ per 10 pairs of salivary glands.

METHODS. β -galactosidase activity was determined spectrophotometrically by following the hydrolysis of chlorophenol-red β -D-galactopyranoside (CRPG)¹⁴. Assay conditions were as follows: 10 pairs of salivary glands dissected from third instar wandering larvae were homogenized in 1 ml of 1 mM MgCl₂ and 50 mM potassium phosphate, pH 7.5. Debris were removed by centrifugation and the supernatant was made 1 mM in CRPG. Reaction mixtures were incubated at 37 °C and the absorbance at 574 nm was monitored during the first 6 h, during which the rate of colour development was constant.

salivary glands of the different lines. As shown in Fig. 3, there is a drastic loss of activity between the transgenic lines 1,453-, 732- and 441-P25-Z and the lines 165-P25-Z, in agreement with the results of the histochemical tests. We interpret the differences between the lines as being due to position effects. Also, we found that lines containing double inserts show a level of β -galactosidase activity that corresponds to the sum of each of the two single insert lines (Fig. 3b). We conclude that there is no limitation on transactivating properties in the transformed lines, and that the level of expression of the transgene depends only on its chromosomal position.

Here we show that although no silk production occurs in *Drosophila*, its salivary glands contain *trans*-acting factors capable of recognizing *cis* elements of a *Bombyx* silk protein gene, and of conferring positional and temporal characteristics on its expression. Thus, although the functions of salivary and silk gland cells have diverged, they probably involve evolutionarily conserved regulatory signals. Our observation that only cells of the anterior region of the salivary gland express the transgene suggests that they are homologous to the cells of the posterior silk gland. To further elucidate conservation of transcriptional regulatory mechanisms, we are determining which of the *cis*-acting elements of the P25 promoter are recognized in *Drosophila* and testing whether genes encoding the silk proteins synthesized in the middle silk gland are also expressed in *Drosophila* salivary glands. □

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Mediation of *Drosophila* head development by gap-like segmentation genes

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THE first phase of embryonic development in *Drosophila* consists of the elaboration and interpretation of maternally encoded information that specifies spatial pattern in the embryo¹. The product of the maternal gene *bicoid* (*bcd*) is thought to organize the anterior pattern of the embryo². Although the *bcd* transcript is localized at the anterior pole of the egg^{3,4} the *bcd* protein forms a stable concentration gradient through the anterior two thirds of the embryo⁵. The graded distribution of *bcd* protein defines position along the anterior–posterior axis of the embryo^{6–8} through the spatially restricted activation of subordinate targets such as the

gap gene *hunchback* (*hb*)^{7–11}. *In vitro* manipulation of specific *bcd* protein binding sites has shown that the gradient of *bcd* protein can in principle define more than one discrete domain of spatially restricted gene activation in the head of the embryo, depending on the affinity of the available binding sites for the *bcd* protein^{8,12}. Genetic analysis has indicated the need for at least one additional zygotic segmentation gene to mediate *bcd* function in portions of the head that lie anterior to the *hb* domain^{2,12–14}. The missing gene activity is expected to be activated in response to higher levels of *bcd* protein than are required for *hb* activation¹². We report here that three previously identified zygotic genes *buttonhead* (*btd*), *empty spiracles* (*ems*) and *orthodenticle* (*otd*)^{15–17} may behave like gap genes that mediate *bcd* function in the embryonic head.

Our analysis of *btd*, *ems* and *otd* was initiated in the search for genes that control expression of the *Distal-less* (*Dll*) gene in the head segments of the embryo. *Dll* function is required for limb development in the embryo^{18,19} and the *Dll* transcript is expressed in the primordia of the limbs from early stages of development²⁰. Previous analysis indicated that *Dll* expression in the thoracic and gnathal segments of the embryo depends on the segment-polarity genes *engrailed* (*en*) and *wingless* (*wg*), but activation of *Dll* expression in the antennal segment does not²⁰. We monitored *Dll* expression in embryos mutant for genes in which the antennal sense organ fails to develop, but in which other *Dll*-dependent structures, like the maxillary sense organ, are normal. In embryos mutant for three such genes—*btd*, *ems* and *otd*—*Dll* is not expressed in the primordium of the antenna, but is expressed in the primordia of the other limbs (Fig. 1 *a–d*). No defect in *Dll* expression was observed in embryos mutant for a fourth gene, identified by a single allele called XQ (ref. 15; data not shown).

Careful examination of the cuticular phenotypes of *btd*, *ems* and *otd* mutants indicated that these genes are required in overlapping but non-identical domains in the embryonic head (Fig. 2). This analysis suggests that *btd* activity is required for development of mandibular, intercalary and antennal structures (Fig. 2, *d–f*). The activity of *ems* is required for development of intercalary and antennal structures and portions of the head skeleton from the pre-antennal region¹⁷ (Fig. 2*g–i*). Some mandibular structures lacking in *btd* mutants are present in *ems* mutants, suggesting that although they overlap to some extent, *ems* acts more anteriorly. Similarly, *otd* mutant embryos lack antennal and pre-antennal structures (Fig. 2 *j–l*). Embryos mutant for *otd* develop structures from the intercalary segment that are defective in *ems* mutants and lack more of the pre-antennal head structures than do *ems* mutant embryos, suggesting that *otd* is required in a more anterior domain than is *ems*.

As structural defects in the head skeleton are often difficult to interpret and not all structures can be scored unambiguously (see legend to Fig. 2), we monitored the effects of the mutants on the expression of *en* and *wg*, which label individual segments of the embryonic head in very characteristic patterns^{21,22} (Fig. 1, *e, i*). Consistent with the results of the cuticular analysis, the mandibular, intercalary and antennal segments are absent in the *btd* mutant embryo. Similarly, the intercalary and antennal segments and a portion of the pre-antennal region defined by the *en* head spot are deleted in *ems* mutant embryos. The antennal segment and a greater portion of the pre-antennal region, including the region expressing *wg*, are deleted in *otd* mutant embryos. Mutations in each of the genes cause the deletion of adjacent segments in the head (presented schematically in Fig. 3). The domains in which *btd*, *ems* and *otd* act, overlap in the antennal segment but each is out of phase with its neighbour(s) by one segment. There is no indication of homeotic transformation of the identities of the affected segments, rather these segments are absent. Therefore *btd*, *ems* and *otd* seem to be required to establish contiguous blocks of segments. In this respect their function is similar to that of the gap genes like *hb*, *Krüppel* and *knirps* (*kni*), which specify overlapping domains in the embryonic trunk.

If the *btd*, *ems* and *otd* genes serve as zygotic interpreters of the maternal positional information in the head, we expect that their activity should be required during early embryogenesis, as for the gap genes. The phenotypic criteria that suggest a gap gene-like role for *btd*, *ems* and *otd* are based on morphological and molecular markers expressed at a relatively late stage of development. We therefore sought indications that these genes might function early. The *btd* gene can be shown to function at the blastoderm stage in two independent ways. First, the pattern of *Dll* expression is altered in *btd* mutant embryos during cellularization of the blastoderm (Fig. 4a, b). Second, the activity of *btd* is required for the activation of the anterior ring of expression of the gap gene *kni* at the cellular blastoderm stage (data not shown). This *kni* stripe lies just anterior to the presumptive cephalic furrow, in what will be the mandibular segment. The activity of *ems* is not required for activation of the *kni* stripe, even though most of the cells in the *kni* stripe also express *ems* (data not shown). These observations are

consistent with the suggestions, based on the later phenotypes, that *btd* is required for development of the presumptive mandibular segment and that the posterior limit of the domain of *ems* action is anterior to that of *btd*. Although the pattern of *btd* expression is not known, the domain in which it appears to be required at the blastoderm stage is consistent with the later requirement in the mandibular, intercalary and antennal segments.

Both *otd* and *ems* exhibit many of the features that would be expected of genes that fulfil the necessary *bcd*-dependent, gap gene-like function in the head: *ems* is first expressed during cellularization of the blastoderm in a single stripe in the head that corresponds to the locations of the primordia of the structures affected in mutant embryos¹⁷; the *otd* gene product is expressed in a somewhat broader stripe in the head during cellularization³². Expression of both genes depends on *bcd* and the position at which their respective stripes are expressed along the anterior-posterior axis is dependent on *bcd* copy number

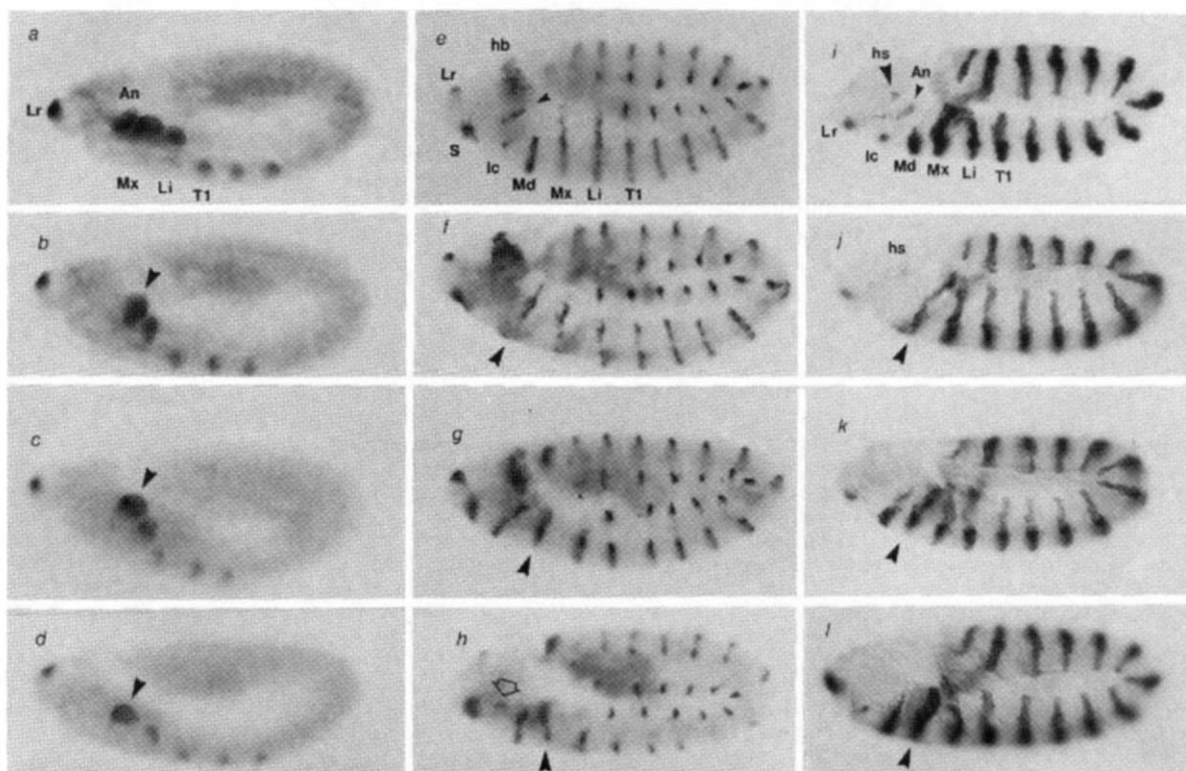


FIG. 1 Embryos mutant for *btd*, *ems* and *otd* exhibit phenotypes characteristic of gap genes. a–d, *Distal-less* expression in wild-type, *btd*, *ems* and *otd* embryos, respectively. Embryos mutant for the three genes lack *Dll* expression in the primordium of the antenna. Arrowhead indicates the maxillary limb primordium. For a detailed description of the *Dll* expression pattern see ref. 20. e–h, *wingless* and *i–l* *engrailed* expression in wild type, *btd*, *ems* and *otd* embryos, respectively. The patterns of *wg* and *en* expression are very characteristic in the head segments^{21,22}. Each segment can be clearly identified, as indicated in e and i. Embryos mutant for *btd* lack expression of both *wg* and *en* in the mandibular, intercalary and antennal segments. The maxillary segment (large arrowhead) is unaffected. The pre-antennal region of the head is not affected, as monitored by the pre-antennal head spot of *en* (hs) and the head blob of *wg* expression (hb). The deletion seems to be in segmental, rather than parasegmental, register as *en* and *wg* stripes are always affected together in a given segment. Embryos mutant for *ems* lack expression of both *wg* and *en* in the intercalary and antennal segments and in a portion of the pre-antennal region of the head. Note that the pre-antennal head spot of *en* is missing, but that the *wg* head blob is still present. Expression of both genes is present in the mandibular segment. Embryos mutant for *otd* lack expression of both *wg* and *en* in the antennal segment and in the pre-antennal regions of the head defined by both *en* and *wg* expression. Both genes are expressed in the

mandibular and intercalary segments. Open arrow in h, intercalary expression of *wg*. Abbreviations; T1, first thoracic; Li, labial; Mx, maxillary; Md, mandibular; Ic, intercalary; An, antennal segments; hs, the head spot of *en* expression; hb, the more anteriorly located head blob of *wg* expression; Lr, labrum; S, stomodeum. The small arrowhead in e indicates the antennal segment.

METHODS. *Dll* and *wg* expression were visualized by whole-mount *in situ* hybridization²⁸, modified as described²⁰. Expression of *en* was detected by labelling with a monoclonal antibody (kindly provided by P. A. Lawrence) as described²⁹. The *btd* gene is defined by 3 mutants (designated XO, XA, XG and is included in a deficiency Df(1) C52, see ref. 16). As no duplication exists that covers the locus, these lesions have not been directly tested for allelism. All of the putative alleles including the Df, map genetically (to position 31) into the small interval between *almondex-lozenge* and *vermillion* (map positions 27.7 and 33.0 respectively). XO and XG produce a strong phenotype, indistinguishable from the Df. XA produces a weaker phenotype in which mandibular segment is often not affected. The cytogenetic location of *btd* is delimited to the region 8E4–9A1 by Df(1) C52 (8E4; 9C) and the duplication Dp(1; 2) v^{+75d} (9A2–10C; 40–41), which does not include *btd*. The *ems* alleles 9H and 9Q encode non-functional proteins in which termination codons occur before the homeodomain and therefore show the complete lack of function phenotype¹⁷. Both a deletion (Df(1) KA14) and a point mutant allele (YM) of *otd* (ref. 15) exhibit the same phenotype.

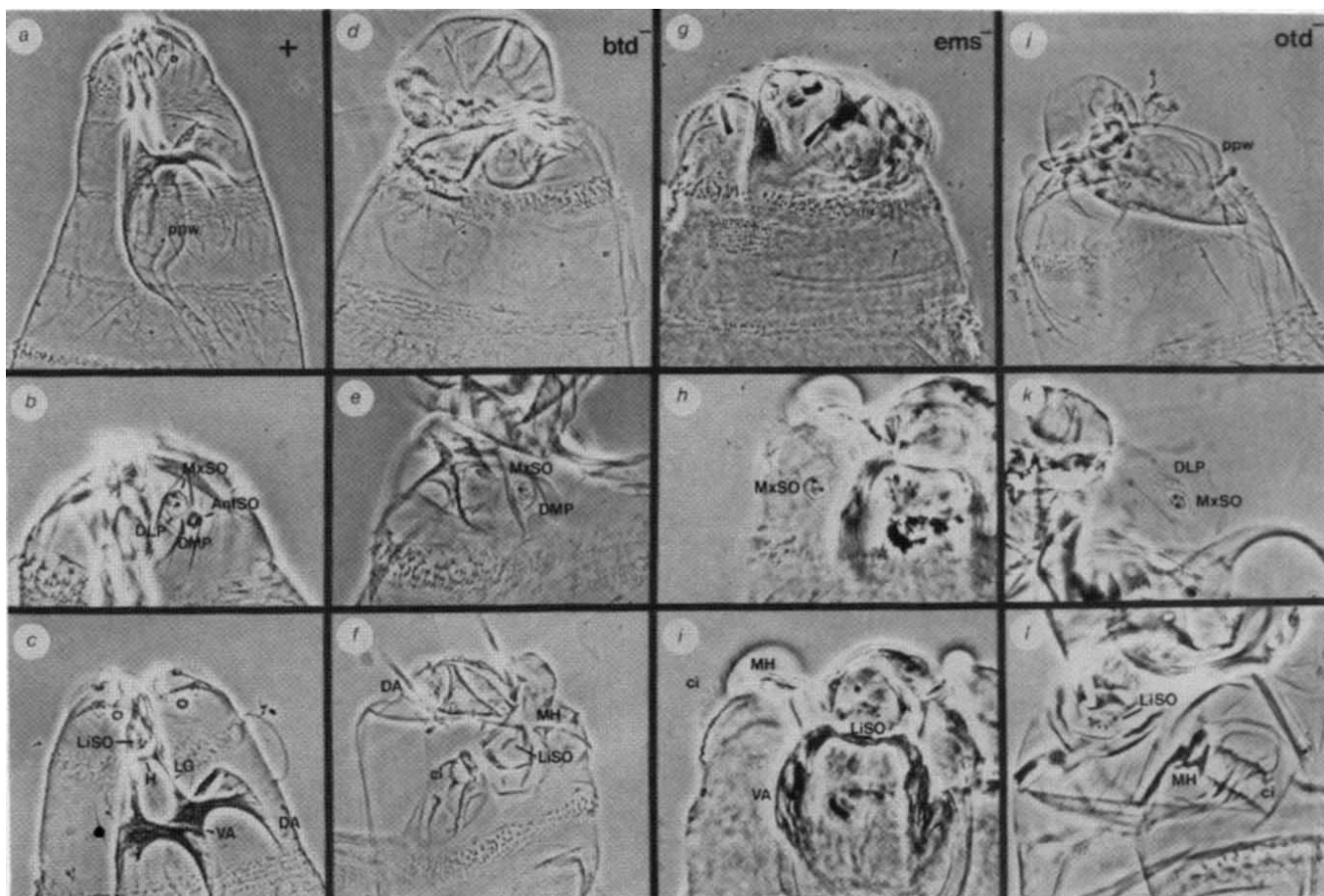


FIG. 2 The genes *btd*, *ems* and *otd* affect overlapping but distinct regions of the head. Cuticular phenotypes of the head region of wild-type (a–c), *btd* (d–f), *ems* (g–i) and *otd* (j–l) embryos. The segmental origin of the structures of the embryonic head have been determined by ultraviolet-laser-ablation fate mapping³⁰. Structures derived from the labial and maxillary segments are present in all the mutants. Labial sense organ (LiSO), cirri (ci), mouth hooks (MH), Maxillary sense organ (MxSO). The labrum is also present in all mutants, but is difficult to recognize in embryos where head involution has failed, as are other elements of the head skeleton from the labial segment. Embryos mutant for *btd* lack the ventral arms (VA), of mandibular origin. The Intercalary segment contributes the posterior wall of the pharynx (ppw), which is reduced in *btd* (not shown). The antennal sense organ (AntSO) is missing. The dorsal arms (DA) of the head skeleton, which derive from the pre-antennal region, are present. The dorso-medial papilla is present (DMP). This structure was originally described as being of antennal

origin, as fate mapping studies place it close to the antenna. The present data suggest that the DMP may be of pre-antennal origin, possibly deriving from a position close to the *en* head spot as the DMP is missing in *ems* mutant embryos. Similarly the dorso-lateral papilla (DLP) is present in *otd* but absent in *ems*, which suggests that the DLP might be intercalary in origin, rather than mandibular as proposed previously³⁰. These ambiguities in the previously proposed segmental origins lie well within the experimental uncertainty of the fate mapping experiments, as all of the structures map very close together near the boundary region between the head segments (see Fig. 8 in ref. 30). Mandibular structures like the VA are present in *ems* and *otd*, but such structures are difficult to identify clearly in *otd* because of the more extreme defects in head involution. Note that intercalary structures such as the ppw develop normally in *otd* mutant embryos (compare a and j).

(ref. 17, and R. Finkelstein *et al.*, personal communication). The suggestion that *btd*, *ems* and *otd* might be direct targets of *bcd* protein is strengthened by the observation that *bcd* protein expressed in salivary glands binds to the cytogenetic loci where the *ems* and *otd* genes are located (W. Driever and C. Nüsslein-Volhard, personal communication). It remains to be determined whether *bcd* directly activates any of these genes in a concentration-dependent manner. The fact that both *ems* and *otd* encode homeobox-containing proteins^{17,32} is consistent with their proposed function as gap-like segmentation genes required to promote the development of head segments.

Several lines of evidence suggest that the process of subdivision of the head into metameric units may differ fundamentally from that of the trunk. In the trunk, combinatorial inputs from the gap genes establish the domains of pair-rule gene expression^{23,24}, which in turn define the metameric patterns of segment-polarity gene expression²⁵ (reviewed in refs 26, 27). In the head, *btd*, *ems* and *otd* seem to function like gap genes

as they are required to specify broad overlapping domains. However, the segmentally repeated patterns of *en* and *wg* are established in this region without input from pair-rule genes. One scenario that might explain the generation of a segmented pattern in the head is based on the observation that the posterior limits of the domains of action of *btd*, *ems* and *otd* are out of phase by one segment. It is possible that segmentation of the head is specified directly by a combinatorial input of information from these—and possibly other—as-yet-unidentified genes. This suggestion derives in part from the observation that although the *btd*, *ems* and *otd* domains overlap considerably, we were unable to demonstrate any hierarchical relationship among them. The expression of *ems* is not obviously affected in either of the other two mutants (data not shown). If *btd*, *ems* and *otd* are direct mediators of the anterior positional information encoded by the maternal *bcd* protein gradient, they might accomplish simultaneously what the gap and homeotic selector genes do in the trunk of the embryo. □

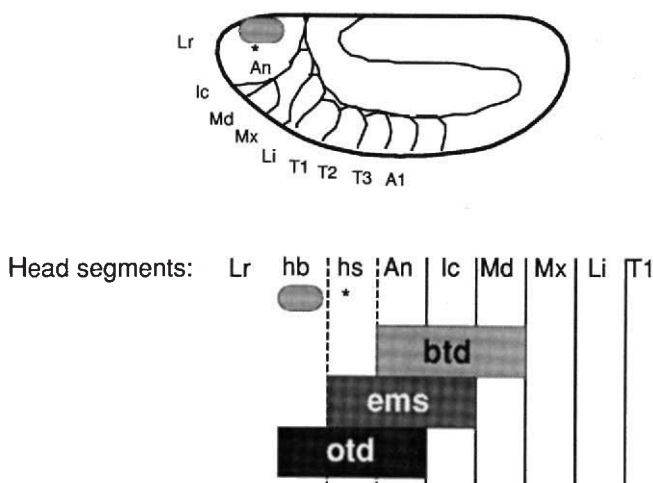


FIG. 3 Schematic representation of the segmental defects in the mutants. The *btd* gene is required for development of the mandibular, intercalary and antennal segments, but not for maxillary and pre-antennal regions. The *ems* gene is required in intercalary and antennal segments and in a part of the pre-antennal domain—defined by the *en* head spot (hs)—but not in more anterior pre-antennal regions—defined by the *wg* head blob (hb)—or in the mandibular segment. The *otd* gene is required in the antennal segment and both pre-antennal regions, but not in intercalary or mandibular segments. The domains in which the genes are required overlap and are out of phase by one segment at their posterior limits. The anterior limit of the domain of action of *btd*, *ems* and *otd* are also out of phase, occupying progressively greater portions of the pre-antennal region. Their effects on *en* and *wg* expression anteriorly may reflect cryptic segmentation in the pre-antennal region of the head. These domains (hs and hb) are indicated with dashed lines to point out that they may not be true segments. Jürgens *et al.*³⁰ and Struhl³¹ both propose six head segments. None of the genes analysed here affects the labral segment (Lr).

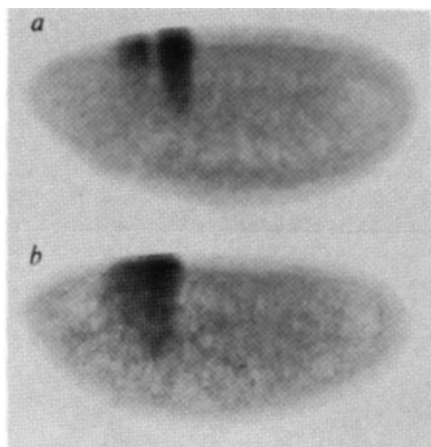


FIG. 4 The pattern of expression of *Dll* is altered at the blastoderm stage in *btd* mutant embryos. *Dll* is first expressed during cellularization of the blastoderm, in two bands that are restricted in their dorsal-ventral extents²⁰. Double-labelling with probes that detect *Dll* and *wingless* shows that the more posterior band corresponds to the primordium of the maxillary and labial limbs (which resolves at a later stage into discrete primordia; data not shown). Following the development of the mature pattern of *Dll* expression suggests that the anterior band corresponds to the presumptive antennal primordium²⁰. In *btd* mutant embryos only one broadened stripe of *Dll* expression is seen at the blastoderm stage. As the mandibular, intercalary and antennal segments are absent at later stages in *btd* mutant embryos, it seems likely that the broadened *Dll* band observed reflects a deletion of the early antennal primordium with a concomitant anterior expansion of the maxillary-labial stripe. In the absence of hybridization probes that distinguish these primordia at the blastoderm stage, this suggestion cannot be directly tested.

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The orthodenticle gene is regulated by *bicoid* and *torso* and specifies *Drosophila* head development

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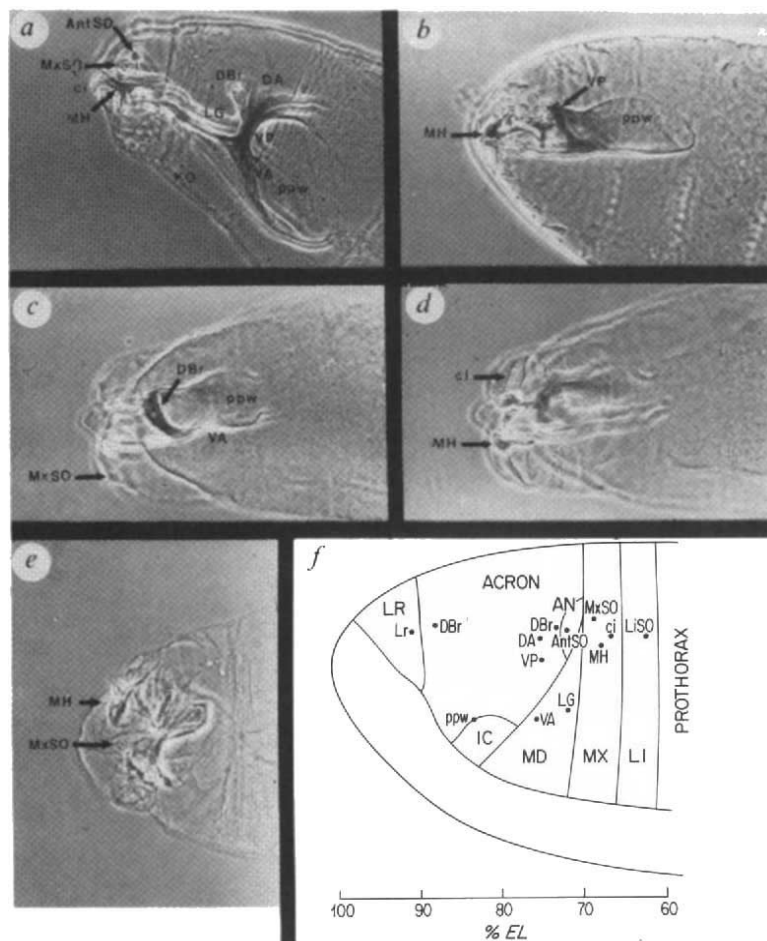
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IN the *Drosophila* embryo, cell fate along the anterior–posterior axis is determined by maternally expressed genes^{1,2}. The activity of the *bicoid* (*bcd*) gene is required for the development of larval head and thoracic structures³, and that of maternal *torso* (*tor*)¹ for the development of the unsegmented region of the head (acron). In contrast to the case of thoracic and abdominal segmentation^{4,5}, the hierarchy of zygotically expressed genes controlling head development has not been clearly defined. The *bcd* protein, which is expressed in a gradient⁶, activates zygotic expression of the gap gene *hunchback* (*hb*)^{7,8}, but *hb* alone is not sufficient to specify head development. Driever *et al.*⁹ proposed that at least one other *bcd*-activated gene controls the development of head regions anterior to the *hb* domain. We report here that the homeobox gene *orthodenticle* (*otd*), which is involved in head development, could be such a gene. We also show that *otd* expression responds to the activity of the maternal *tor* gene at the anterior pole of the embryo.

Mutations at the *otd* locus cause embryonic lethality, head involution fails to occur properly and several larval cuticular and sensory head structures are deleted or grossly perturbed¹⁰. The deleted elements are antennal or pre-antennal in their segmental origin (Fig. 1). Structures corresponding to the labral, intercalary and gnathal (mandibular, maxillary, and labial) segments develop, but are often disrupted, probably as a result of abnormal head involution. No evidence for homeotic transformation or duplication of head structures is found in *otd* embryos. Analysis of the cuticular phenotype of the head, which

FIG. 1 Head defects in *otd* embryos. *a-e*, Phase contrast micrographs of cuticular preparations of wild-type (*a*) and *otd* mutant embryos (*b-e*). For abbreviations, see below. For a detailed description of wild-type head cuticular structures and sense organs, see ref. 24. Anterior is to the left in all panels. *b* and *c*, VA and ppw, which are of mandibular and intercalary origin respectively. The DBr and VP, additional elements of the cephalopharyngeal skeleton, are also present. The DA, which are derived from the pre-antennal region, are either absent or fragmented. *d*, Derivatives of the maxillary segment, the cirri and mouth hooks, are present in *otd* embryos. *e*, Absence of the AntSO and the presence of the MxSO; the LiSO is also present (not shown). The dorsal-medial papilla, which is probably pre-antennal in origin (S. Cohen and G. Jurgens, personal communication) is deleted (not shown). *f*, Fate map of the embryonic head at the blastoderm stage (adapted from Jurgens *et al.*²⁴). Segmental assignment is based on the defects produced by laser irradiation at this stage of development. The labral, antennal, intercalary, mandibular, maxillary and labial segment boundaries are demarcated, and the fate map positions of several head structures indicated. Abbreviations: antennal sense organ, AntSO; antennal segment, AN; cirri, ci; dorsal arms, DA; dorsal bridge, DBr; egg length, EL; intercalary segment, IC; Keilin's organ, KO; labial sense organ, LiSO; labial segment, LI; labral segment, LR; labrum, Lr; lateralgrate, LG; mandibular segment, MD; maxillary segment, MX; maxillary sense organ, MxSO; mouth hooks, MH; posterior wall of the pharynx, ppw; ventral arms, VA; vertical plate, VP.

METHODS. Cuticles were prepared as described previously²⁵. The cuticular phenotypes shown are similar for two ethylmethane sulphonate-induced mutant alleles, *otd*^{XDB7} and *otd*^{YH13}, and one deficiency allele, *otd*^{JA101} (refs 10, 30).

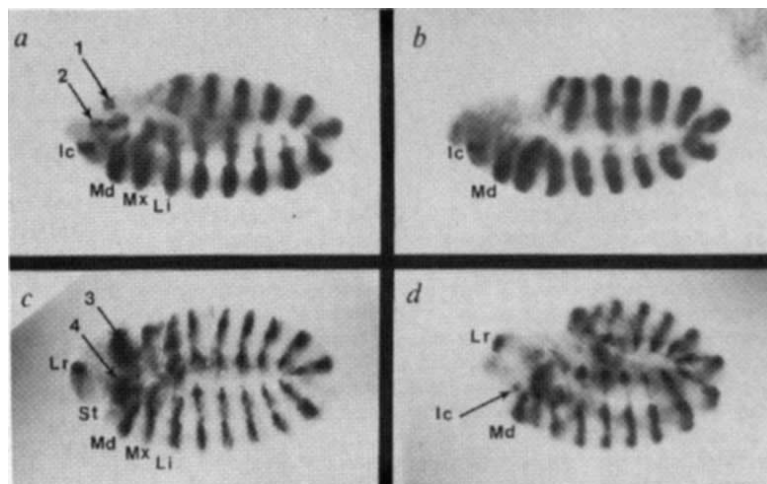


is difficult in first instar larvae, is supported by the expression patterns of the segment polarity genes *engrailed* (*en*) and *wingless* (*wg*) in the developing embryo (Fig. 2*a, c*). In addition to the reiterated stripes marking the gnathal, thoracic and abdominal segments, each of these genes is expressed in a specific, segmental pattern in more anterior head regions (refs 11-13; S. Cohen, personal communication). In *otd* mutant embryos, the expression of *en* and *wg* in antennal and pre-antennal regions is deleted (Fig. 2*b, d*). Consistent with the cuticular *otd* phenotype, expression corresponding to the labral, intercalary and gnathal segments is present in *otd* embryos.

FIG. 2 Specific elements of *engrailed* (*en*) and *wingless* (*wg*) head expression are deleted in *otd* mutant embryos. *a* and *b*, Expression pattern of *en* at the germ band-extended stage in wild-type and *otd* embryos, respectively. In wild-type embryos, *en* is expressed anterior to the gnathal segments (Md, Mx and Li) in the intercalary (Ic) and antennal (arrow 2) segments (ref. 11; S. Cohen, personal communication). In addition, a pre-antennal spot of expression (arrow 1) is visible. In *otd* embryos, the antennal and pre-antennal regions of *en* expression are deleted, but expression in the intercalary and gnathal segments is retained. *c* and *d*, Expression pattern of *wg* in wild-type^{12,13} and *otd* embryos, respectively. Expression in the stomodeal (St), labral (Lr), antennal (arrow 4) and pre-antennal (arrow 3) regions is visible. Expression in the intercalary segment is present, but cannot be seen in this focal plane. In an *otd* embryo (*d*), the deletion of the regions of antennal and pre-antennal staining can be seen. Expression of *wg* marking the Ic and Lr segments is retained. In all panels, anterior is to the left and dorsal at the top.

METHODS. Expression of *en* and *wg* were monitored using two *lacZ* insertion strains that accurately reproduce the embryonic patterns of protein expression of *en* (C. Hama and T. Kornberg, personal communication; ref. 26) and *wg* (N.P. and J. Kassis, unpublished observations). Females heterozygous for *otd* mutations were crossed with

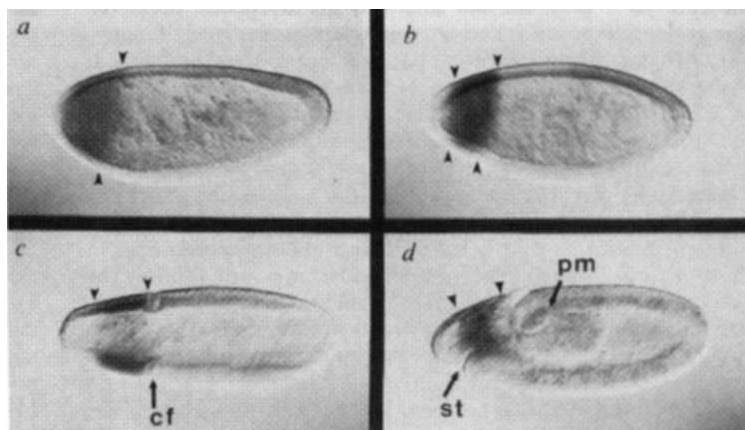
To determine the relationship between the head phenotype observed in mutant embryos and *otd* expression, we analysed the early pattern of *otd* transcription (Fig. 3). Transcription is evident before cellularization at stage 4 of embryonic development and is restricted to a circumferential anterior region extending to the pole of the embryo. By the time cellularization is complete, *otd* expression has retracted from the pole and is confined to a broad circumferential stripe extending from 70 to 90% of the egg length, which subsequently diminishes in intensity ventrally. Expression is also detectable in the yolk nuclei within the region of the stripe (data not shown). The



males from either *lacZ* strain. The *lacZ* expression was detected using an antibody to β -galactosidase with processing as previously described²⁶.

FIG. 3 Early expression of *otd* during embryogenesis. In all panels, anterior is to the left and dorsal at the top, except for *c* which is a dorsal view. Small arrows indicate the anterior-posterior boundaries of the domains of *otd* transcription. *a*, Expression of *otd* in a late stage-4 embryo; transcription can be seen in the anterior region. *b*, Cellularized stage-5 embryo; *otd* expression has retracted from the anterior pole, and forms a circumferential stripe extending from 70 to 90% egg length. Transcription diminishes in the ventral region of the stripe during subsequent stages. *c*, During early gastrulation in stage-6 embryos, the cephalic furrow forms just posterior to the domain of *otd* transcription. In germ band-extended embryos (stage 8), *otd* expression is localized to the procephalic head region. Staging of embryos was according to Campos-Ortega and Hartenstein²⁷. Abbreviations: cephalic furrow, cf; stomodeum, st; posterior midgut, pm.

METHODS. *In situ* hybridization to whole-mount embryos was performed according to Tautz and Pfeifle²⁸. The probe used for digoxigenin-labelling was a 3.8-kilobase *otd* complementary DNA (ref. 30).



formation of the cephalic furrow occurs just posterior to the domain of *otd* expression. By the extended germ-band stage, *otd* transcription is localized within the head region.

We have previously characterized the *otd* gene and shown that it encodes a predicted homeodomain protein³⁰. Homeodomains can be classified according to the amino acid at position 9 of the putative recognition helix, which has been shown to confer binding specificity^{14,15}. The *otd* and *bcd* gene products^{16,17} are the only known proteins with a lysine residue at this position of the homeodomain.

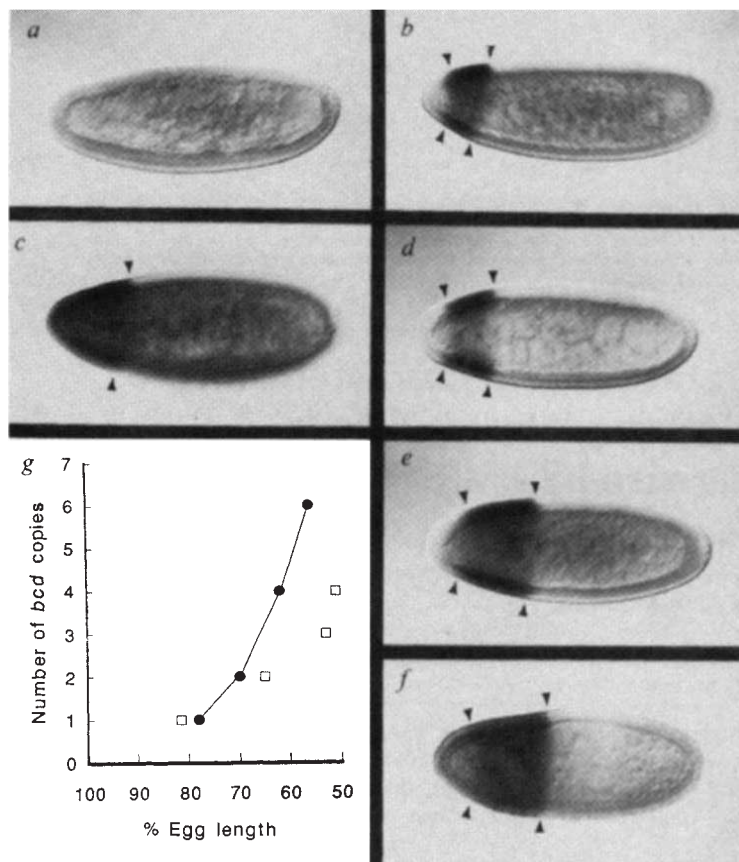
The expression of *otd* is positively regulated by *bcd* in a concentration-dependent fashion (Fig. 4a-g). In embryos lacking maternal *bcd* product, *otd* transcription is absent. As the number of copies of *bcd* increases, the domain of *otd* transcription extends progressively towards the posterior end of the embryo. The expression of *otd* is negatively regulated by *tor*;

in the absence of maternal *tor* activity, *otd* transcription fails to retract from the anterior 10% of the embryo (Fig. 4c). A similar effect has been observed for zygotic *hb* expression⁹.

As the effect of *hb* mutations on head development¹⁸ does not account for the absence of all head structures in *bcd* embryos the existence of an additional *bcd*-regulated gene(s) has been proposed⁹. Driever *et al.*⁹ postulated the existence of a gene(s) which would be activated by higher *bcd* concentrations than is *hb* and specify all or part of the anterior head region. The predicted domain of expression of such a gene would therefore be localized within the anterior region of the *hb* domain of transcription. The *otd* gene, which is required for the development of structures from the antennal and pre-antennal head segments, fulfills these criteria. In addition to *otd*, the gene *empty spiracles* (*ems*)¹⁹, which is also required for head development, is expressed in a circumferential stripe in the anterior region of

FIG. 4 Expression of *otd* is regulated positively by *bcd* and negatively by *tor*. *a-f*, *In situ* hybridization to embryos of various maternal genotypes. Anterior, left; all views are dorsal except *b*, which is lateral. *a, b, d, e* and *f*, Embryos derived from mothers carrying 0, 1, 2, 4, and 6 copies, respectively, of the wild-type *bcd* gene. The posterior extent of *otd* transcription is progressively extended as *bcd* dosage increases; *g* depicts this shift graphically. The *otd* points (●) indicate the position of the posterior limit of *otd* expression as a function of maternal *bcd* dosage. In all cases, measurements were made using dorsal views. In addition, the effect of maternal *bcd* dosage on *bcd* protein expression is shown. The *bcd* points (□) indicate the egg length position at which the same level of *bcd* protein is found for embryos derived from females with varying *bcd* dosages. The 65% value of *bcd* immunostaining intensity was chosen arbitrarily. Data is derived from ref. 29. *c*, Expression of *otd* in an embryo lacking maternal *tor* product; *otd* expression fails to retract from the anterior pole at the cellular blastoderm stage. Arrowheads in all panels show the anterior and posterior position of the domain of *otd* expression.

METHODS. *In situ* hybridization was as in Fig. 3. The genotypes of the females that produced embryos with 0, 1, 2, 4 and 6 copies of *bcd*⁺ were respectively: *bcd*^{E1}/*bcd*^{E1}, *bcd*^{E1}/±, ±/±, *BB9* + *BB16*/± and *BB9* + *BB16*/*BB9* + *BB16*. The *BB9* + *BB16* chromosome contains three copies of a wild-type *bcd* gene (G. Struhl, personal communication). The *tor* mutant embryos were derived from *tor*^{wk34}/*tor*^{wk34} homozygous females¹.



the embryo²⁰. Both the *otd* and *ems* genes encode predicted homeodomain proteins and are dependent on the level of maternal *bcd* gene product for the establishment of their domains of expression. Mutations at each of these loci cause the deletion of subsets of adjacent head segments and show no evidence for the homeotic transformation of structural elements. If these genes are indeed directly transcriptionally activated by *bcd* (as suggested by the early appearance of their expression), then the regulation of *Drosophila* embryonic head development may be different from that of the thoracic and abdominal regions. In those regions, the earliest zygotic regulation is provided by the gap genes, several of which encode zinc-finger-containing proteins^{21–23} required for the sequential activation of the downstream pair-rule and segment polarity genes. In the head, however, homeobox genes such as *otd* and *ems* may function as direct intermediaries between the *bcd* morphogen and the segment polarity genes. □

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Detection and characterization of a folding intermediate in barnase by NMR

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PROTEIN engineering is being developed for mapping the energetics and pathway of protein folding. From kinetic studies on wild-type and mutant proteins, the sequence and energetics of formation of tertiary interactions of side chains can be mapped

and the formation of secondary structure inferred^{1,2}. Here we cross-check and complement results from this approach by using a recently developed procedure that traps and characterizes secondary structure in intermediate states using ¹H NMR^{3,4}. The refolding of barnase is shown to be a multiphasic process in which the secondary structure in α -helices and β -sheets and some turns is formed more rapidly than is the overall folding.

It is desirable to corroborate the results of the novel procedure of detecting and characterizing an intermediate in the refolding of barnase by protein engineering methods², with a second technique. The method of choice is the NMR-hydrogen-exchange procedure of Udgaonkar and Baldwin³ and Roder et al.⁴ because it can detect individual interactions as well as gross structure. Their procedure is based on the observation that protons that are in NH...O=C hydrogen bonds are protected to varying extents against exchange with solvent^{3,4}. Samples are taken from a reaction mixture in which a deuterated protein is being refolded in D₂O and NMR is used to examine which of the ND groups are in rapid exchange and which are protected. By this means, it was shown that the repeating secondary structure in regions of β -sheet in RNase A (ref. 3) and α -helices in cytochrome *c* (ref. 4) are formed faster than is the final folded structure.

The refolding of barnase was first monitored by the change in the fluorescence of its three tryptophan residues under identical conditions to those used in the NMR experiments (Fig. 1, 1.3 M (deuterated) urea, 99.8% D₂O, pD 6.3 at 25 °C). There is a fast phase accounting for 80% of the reaction, which has a half life of about 140 ms, and a slow phase of 20%, which is 50 times slower with a half life of 8.5 s. The latter phase corresponds to the interconversion from *cis* to *trans* of the fraction of proline residues that equilibrate in the unfolded enzyme—the native enzyme has three proline residues that are all *trans*^{1,2}. There is some hint that the fast phase could be resolved into two phases of rate constant 11–16 s^{−1} and 4.6 s^{−1} (Fig. 1).

NMR experiments were made possible by assigning more than 99% of the proton resonances in the ¹H NMR spectrum of barnase to their amino-acid residues⁵. Some 45 of the backbone NH protons in the folded protein undergo slow exchange with solvent because they are involved in hydrogen bonds in secondary and tertiary structure. Barnase was denatured and all exchangeable NH groups deuterated (>90% exchange) by incubating in 6.5 M (deuterated) urea and 99.8% D₂O at pD 6.3. The denatured and deuterated protein was allowed to refold by diluting fivefold into D₂O at 25 °C. Samples were taken during the refolding process and exposed for 5–15 s to a labelling pulse of H₂O buffered at pH 8.5 where there is fast exchange of unprotected ND deuterons ($t_{1/2}$ ~ 5 ms; refs 3, 4). The pH was lowered to 3.5, where exchange is very slow, and the fraction of H-exchange measured by two-dimensional (2-D) NMR.

It is found (Fig. 2) that 29 of the positions are sufficiently protected under the experimental conditions that the change in protection with time may be followed. Three of the positions, ND(H)s of Ile 25, Asn 77 and Ser 50, are protected according to a time course that is essentially identical to that of the overall refolding process (Fig. 3). Defining the fraction of protons in rapid exchange at zero time as 1.0, compared with 0 when protected in the fully folded protein, the amplitude of the kinetic changes is 0.74–0.76. The theoretical maximum amplitude for the fast phase is 0.8, as 20% of the protein folds slowly, limited by the proline isomerization. Significantly, Ile 25, Asn 77 and Ser 50 make tertiary hydrogen bonds that are not within regular secondary structure but are a consequence of the overall fold of the molecule. It is found, however, that several of the deuterons in the regular secondary structure become protected much faster (Fig. 3 and Table 1). The time courses for many of these are at least biphasic, the slower phase varying from 10–28 s^{−1}, with an amplitude of 50–80% of the maximum. The fastest phase(s) is generally complete by 5 ms and has a smaller amplitude of 10–50%. Multiphasic exponential curves are notoriously

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difficult to analyse as, unless the data are exquisitely accurate, the sum of two or more exponentials with time constants differing by a factor of two can usually be fitted to a single exponential. But simple inspection of the results shows clearly and unambiguously that extensive secondary structure is formed on a time scale that is shorter than that for the overall folding.

The question has been raised as to whether the multiphasic kinetics results from a collection of parallel pathways rather than from a sequential mechanism of reaction, that is, the kinetics is simply a consequence of a heterogeneous population of unfolded molecules, each folding at its own rate (equation (1), where ΣE_U is the set of unfolded states that forms the folded protein E_F , by a set of parallel first-order rate constants, Σk). It can be difficult to distinguish



between parallel and sequential pathways when observing just the overall macroscopic properties of a protein. But this can be done when observing individual sites, as in hydrogen exchange. The kinetics of formation of fully folded protein proceeding by

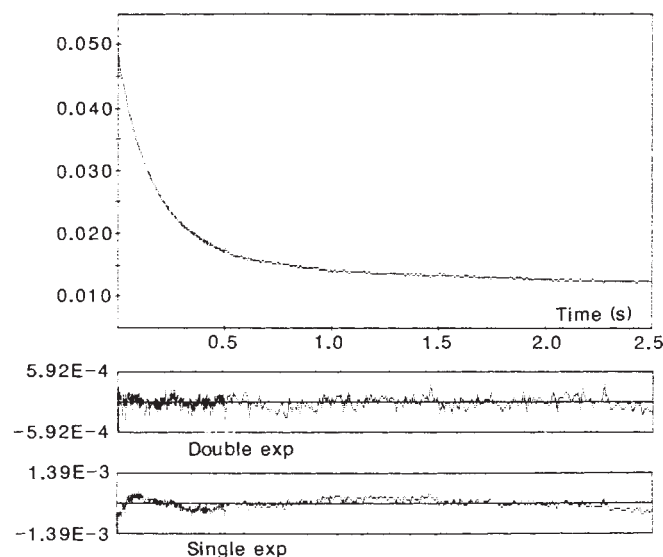


FIG. 1 Time courses for refolding of barnase in D_2O , 100 mM MES buffer (pD 6.3), 1.3 M urea at 25 °C, monitored by regain of tryptophan fluorescence, measured in an Applied Photophysics stopped-flow fluorimeter of deadtime 1 ms with excitation at 290 nm and emission at wavelengths greater than 300 nm selected using a cutoff filter. Protein that was acid-denatured in D_2O at pD 1.8 and 1.3 M deuterated urea was mixed 1:1 with a solution of D_2O , 200 mM MES (pD 6.3) and 1.3 M urea at 25 °C, the concentrations of urea being balanced to eliminate Schlieren effects. Twenty three transients were averaged. The lower two panels show the deviation of the data from theoretical curves fitted to either a single exponential for the faster changes (very bottom) or a double exponential. The data seem to fit slightly better a double exponential of rate constants 11.4 and 4.55 s^{-1} , with amplitudes in the ratio 1:3; than a single of 5.1 s^{-1} —the scatter is smaller and seems to be randomly distributed. Refolding kinetics were also performed with a Perkin-Elmer spectrofluorimeter MPF 44B fitted with a stopped-flow mixer². Excitation was at 290 nm and emission at 315 nm. This machine, with monochromators for both excitation and emission, allows emission to be monitored at a single wavelength and also the mixing of high concentrations of urea with water without Schlieren effects, but it has a dead time of 30 ms. Denatured protein in 6.5 M deuterated urea, D_2O , 50 mM MES (pD 6.3) was diluted 11-fold into D_2O , 0.78 M deuterated urea, 50 mM MES (pD 6.3) to give the same final concentrations as for the NMR experiments. Data (not shown) fit equally well a double exponential of rate constants ~ 16 and 4.58 s^{-1} or a single of 5.6 s^{-1} . Repetition of the experiments in H_2O at 0 M urea where renaturation is faster, the conditions used in the calculations by Matouschek *et al.*², showed that the faster phase is lost in the dead time of the stopped-flow machine and so only the slower phase is observed and accurately measured there.

a collection of parallel pathways without any intermediates would be an ensemble of exponential traces each of low amplitude, with the total amplitude being 100%. The same multiexponential time course would apply to the formation of each and every noncovalent bond in the fully folded structure, although the amplitude of the exponential phases would be lower if the noncovalent bond is present to some extent in residual structure in the 'unfolded' state. But we observe a variety of rate constants, varying from clear biphasics on a fast time scale to the tertiary hydrogen bonds involving Ile 25, Asn 77 and Ser 50, which are formed with nearly 100% amplitude of signal in a time course approximating to that of a single exponential. There could be a heterogeneous population of unfolded molecules, but to be consistent with the observed kinetics, they would have to form rapidly a common intermediate before the rate-determining step,

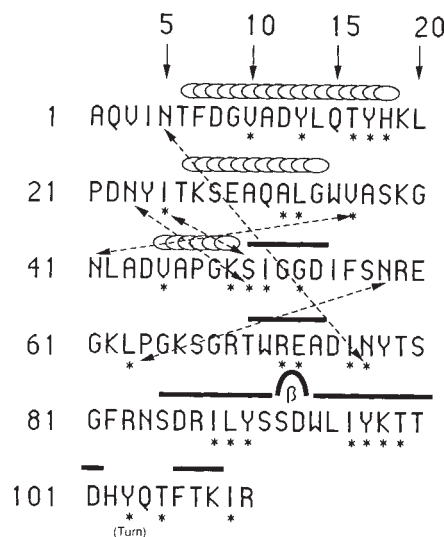


FIG. 2 Backbone $>ND(H)$ s protected from exchange with solvent during refolding. Time courses for the protection of NH groups (ND groups) during the refolding of denatured barnase were determined in 1.3 M deuterated urea, D_2O (pD 6.3) 50 mM MES buffer at 25 °C. Barnase was denatured by incubating in 6.5 M deuterated urea in 99.8% D_2O buffered at pD 6.3 and the NH groups allowed to exchange with the solvent to give ND groups ($>90\%$ exchange). The denatured protein was mixed with four volumes of buffered D_2O to initiate refolding at the same pD using modified versions of the quenched flow or pulsed quenched flow apparatus of Fersht and Jakes⁶. After time intervals from 0 (that is, direct refolding in labelling buffer) and then 6–550 ms, samples were exposed to a labelling pulse of four volumes of H_2O buffered at pH 8.5 (100 mM Tris-Cl buffer) where exchange of unprotected ND deuterons is very rapid. After 5–15 s, which is sufficient time for the all-*trans*-prolyl enzyme to fold fully, the pH was lowered to 3.5 with 500 mM ammonium formate, where exchange is very slow. (The 20% of protein that contains *cis* prolines is fully exchanged in the first fraction of a second in the labelling pulse, in which time $<1\%$ of the proline residues isomerize.) Each sample was concentrated and the buffer changed to D_2O , pD 3.5, with an Amicon MC-8 ultrafiltration cell. Phase-sensitive COSY spectra were recorded at 27 °C on a Bruker AM 500 spectrophotometer⁵. The intensities of the cross peaks in the fingerprint region of the respective residue were determined by measuring peak to peak heights in cross sections parallel to the ω_2 axis⁷. The peak heights were standardized with reference to the cross peak of the non-exchanging aromatic ring protons of Tyr 13. The reproducibility of the data varies according to peak intensity and the degree of overlap. The worst data points analysed are reproducible to within $\pm 10\%$ of maximum peak height, the majority to about $\pm 5\text{--}7\%$, the error being constant throughout each time course. Control experiments on native protein incubated under the conditions of the labelling pulse for several minutes showed that no detectable exchange occurs at the analysed sites. Equilibrium measurements of the unfolding of barnase in D_2O , deuterated urea, 50 mM MES (pD 6.3) and 25 °C were performed as in H_2O ⁸, and the free energy of unfolding found to follow the equation $\Delta G_f = (11.38 - 2.27[\text{urea}])\text{ kcal mol}^{-1}$. Under the conditions of the NMR experiments the equilibrium constant is calculated to be $10^6:1$ in favour of folding.

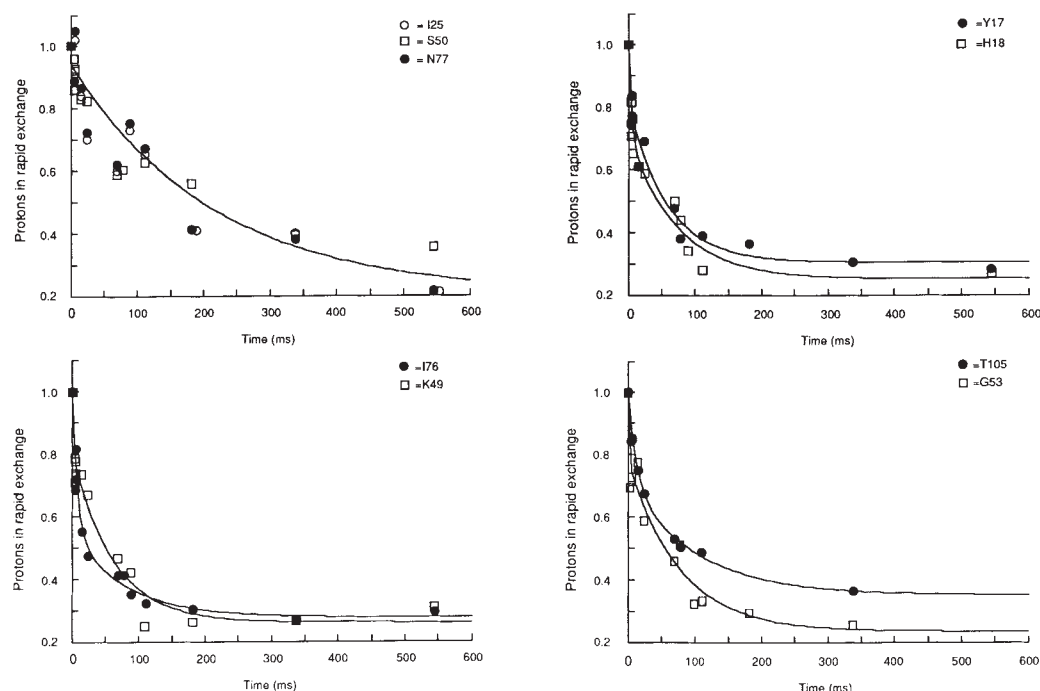


FIG. 3 Time courses for the protection of backbone $>ND(H)s$ against exchange. Conditions as in Fig. 2. Top left, residues Ile 25, Asn77 and Ser50 are protected with a rate constant of 5 s^{-1} , that for the overall folding. The other panels contain representative time courses. The fraction of exchangeable protons is defined as 1.0 at zero time. The expected endpoint for 'complete protection' is 0.2 as 20% of the denatured protein, that fraction containing *cis* prolines, folds with a rate constant of 0.082 s^{-1} , and 550 ms is only 6% of the half life for that step. There is no fast phase in the data for residues Ile25, Asn77 and Ser50 (top left); there is random scatter of time-points compressed in the region 0–7 ms.

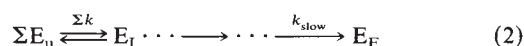
TABLE 1 Protection against hydrogen-deuterium exchange

Residue	Location of $>NH$	Phase 1 Rate constant* (Amplitude) † (s^{-1})	Phase 2 Rate constant* (Amplitude) † (s^{-1})
V10	Helix	>500 (0.31)	28 (0.36)
Y13	Helix	poor data, fast protection	
T16	Helix		18 (0.60)
Y17	Helix	>250 (0.17)	18 (0.52)
H18	Helix	248 (0.29)	14 (0.46)
I25	Tertiary (with S50)		5.8 (0.76)
A32	Helix	poor data, fast protection	
L33	Helix		20 (0.60)
V36	Tertiary (with N41)	>400 (0.27)	3.7 (0.66)
V45	Helix	>400 (0.32)	15 (0.39)
K49	Helix	>500 (0.19)	16 (0.56)
S50	Tertiary (with N23)		4.7 (0.72)
I51	Sheet	>250 (0.22)	13 (0.44)
G53	Sheet	>250 (0.22)	13 (0.54)
L63	turn (with N58)	>250 (0.27)	15 (0.26)
R72	Sheet	>500 (0.52)	15 (0.26)
E73	Sheet	poor data, fast protection	
I76	Sheet	130 (0.44)	12 (0.27)
N77	Tertiary (with N5)		5.8 (0.74)
I88	Sheet		19 (0.53)
L89	Sheet		18 (0.55)
Y90	Sheet		26 (0.48)
I96	Sheet		13 (0.75)
Y97	Sheet	poor data, fast protection	
K98	Sheet		28 (0.60)
T99	Sheet	>500 (0.33)	26 (0.37)
Y103	Turn	250 (0.38)	5.7 (0.49)
T105	end of sheet	80 (0.28)	10 (0.35)
I109	Sheet	>200 (0.19)	10 (0.52)

* The change in protection was analysed by fitting to a two-step exponential of the form $y = A \exp(-k_1 t) + B \exp(-k_2 t) + C$ using the program 'Regression' (Blackwell Scientific Publications, Oxford, UK) which employs the Marquardt algorithm.

† The fraction of protons in fast exchange is defined as 1.0 in the unfolded protein at zero time and in the fully folded protein as 0. The maximum amplitude expected for the phases studied here is 0.8 as 20% of the protein folds on a much longer time scale ($t_{1/2} = 8.5\text{ s}$). The detection of a fast phase that is complete in the dead time of the apparatus, hinges crucially on the value of the point taken at zero time. The method of determining the zero time-point seems valid as the zero-time values for the ND groups of residues 25, 50 and 77—which are relatively slowly protected with a simple exponential time course ($t_{1/2} = 140\text{ ms}$) or a virtually superimposable double exponential time course (see Fig. 1)—are within experimental error of values extrapolated back from 5–7 ms after mixing. Single-letter notation used for amino-acid residues.

and all the molecules pass through this intermediate (equation (2)). The kinetics thus show that there must be at least one intermediate, E_I , formed during folding.



The simplest explanation of the data is that there is a rapid equilibrium set up in the submillisecond timescale in which many of the backbone ND(H)s are protected. The equilibrium constant, which is related to the amplitude of the fast phase for each residue and the degree of protection against the labelling pulse, is a function of the position in the protein. Whether these are abortive complexes or whether the same bonds as in the final structure are formed is not obvious. The helices and sheets are formed from these complexes with a rate constant of $\sim 15\text{ s}^{-1}$. Residue Tyr 103, which is in a bend, and Val 36, which makes a tertiary interaction, are residues that are not in the helices or sheet. They exhibit some of the rapid protection and then take up their final interactions with a rate constant equal to that of the final folding step of $\sim 5\text{ s}^{-1}$. Ile 25, Asn 77 and Ser 50 make their tertiary interactions also at 5 s^{-1} as the structure finally folds.

The NMR H-exchange data confirm the conclusions from the protein engineering studies that the refolding of barnase involves at least one discrete intermediate. Both techniques show that the C-terminal end of the major helix is formed before the main transition state for folding. The NMR data show further structural features, such as early formation of sheet, and definite indications of earlier folding intermediates before the one observed in the protein engineering studies. $\square$

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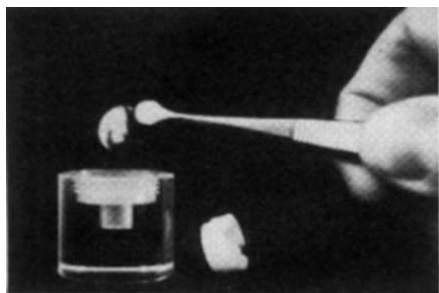
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New product news

Products for the midsummer season include magnetic beads for the isolation of poly(A)⁺ RNA, a mycoplasma testing service and plate-tectonic modelling software for geology buffs.

Genzomed supplies a **microdialysis system** — μ -DIA — for use with small sample volumes (50–200 μ l) of proteins and other macromolecules (*Reader Service No. 101*). The \$25 (US) μ -DIA can be used for desalting, buffer exchange, separation of excess radiolabel, enzyme assays, and the purification of proteins, nucleic acids and other macromolecules.



μ -DIA: Genzomed's small sample volume microdialysis system.

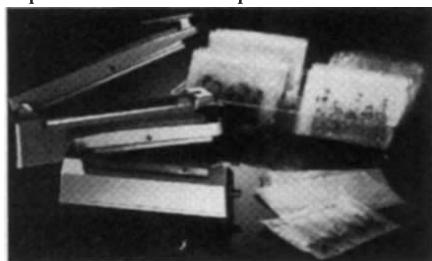
It is available with pre-cut membranes with molecular weight cut-offs of between 500 and 500,000 Da. All kinds of bacteria filters, membrane ultra filters, blotting membranes and dialysis membranes can be used with μ -DIA. Dialysis takes 1–2 hours, Genzomed says, depending on the number of samples and the buffer.

ACCU-SPERM is an **acrosin activity assay** from O.E.M. Concepts that can be used to study subfertility and infertility and which may aid in the evaluation of sperm function (*Reader Service No. 102*). Acrosin can occur in spermatozoa in both inactive and active forms. Proacrosin, the inactive form, represents about 90 per cent of the acrosin in human spermatozoa. The assay uses a membrane-based separation of sperm cells from seminal fluid. At basic pH, proacrosin is converted to enzymatically active acrosin and the total acrosin activity of human spermatozoa is determined. Results are obtained in 90 minutes, says O.E.M., and are read spectrophotometrically by measuring absorbance at 405–410 nm. The \$450 (US) ACCU-SPERM assay kit contains sufficient reagents for 48 assays.

AutoGen 540 is the latest benchtop system for the **isolation of DNA and RNA** from a wide variety of biological sources (*Reader Service No. 103*). The instrument is designed for the rapid isolation of plasmids, cosmids, phage DNA, genomic DNA and RNA from bacterial cultures, tissue suspensions, blood and plant cell

preparations. Two versions are available. The \$36,000 (US) basic version, which has nine reagent positions and is supplied with ten different DNA and RNA protocols on the system's floppy disk. Applications protocols are continually being developed and updated by AutoGen. The more advanced \$42,500 (US) programmable version has 12 reagent positions and, in addition to the ten standard protocols, is fully programmable through an MS-DOS based PC system. According to AutoGen, both versions can process up to 12 samples simultaneously in cycle times of less than 40 minutes. The instrument may be of interest to researchers in the fields of genetic engineering, DNA sequencing, mapping, analysis and as a sample preparation device for DNA probe diagnostics.

Plant micropropagation is a new area for Becton Dickinson and one of the first products off the production line is the Falcon CultuSAK for plant tissue culture (*Reader Service No. 104*). Designed to replace conventional plant tissue culture

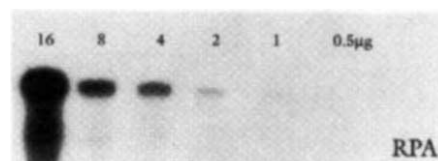
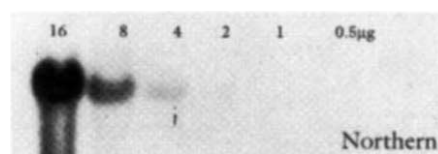


Bagged up and ready to grow.

equipment, CultuSAKs are made of non-porous translucent film that is permeable to gases but, says Becton Dickinson, virtually impermeable to liquids. The system can be sealed completely to reduce the risk of contamination and the problems of desiccation of the culture media. CultuSAKs are available in four sizes to support different types of plant growth.

RNA research

According to Ambion, its Ribonuclease Protection Assay (RPA) kit for the **detection and quantitation of mRNA** is at least ten-times more sensitive than northern blotting (*Reader Service No. 105*). Using high specific activity probes, it is possible to detect as little as 100 femtograms of target RNA after a three-day exposure to X-ray film. Because of the high sensitivity of the assay, low abundance transcripts can be detected without poly(A) selection. Ambion attributes the increased sen-



RNA detection by northern blotting and Ambion's RPA kit. Exposures were 64 hours and 6.4 hours, respectively.

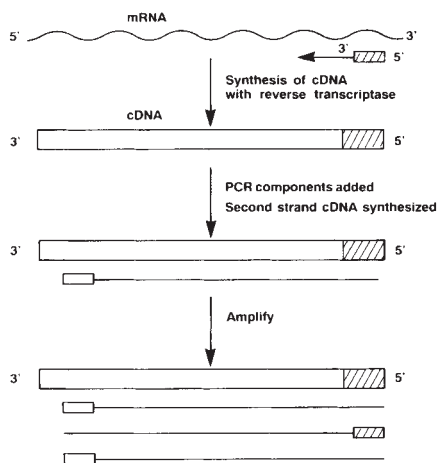
sitivity of its RPA kits to the following: the hybridization reaction occurs in solution, whereas target RNA is bound to a filter with northern blotting; and after RNase digestion, the protected labelled material is separated from digested material by gel electrophoresis, which increases the signal-to-noise ratio. Ambion's \$195 (US) RPA kit contains enough reagents and positive controls for 120 assays. The company also provides *in vitro* transcription kits for RPA probe synthesis using SP6, T7 or T3 RNA polymerases.

Dynal offers Dynabeads Oligo (dT)₂₅ **magnetic beads for the isolation of poly(A)⁺ RNA** directly from solid tissue, cell lines and plants (*Reader Service No. 106*). The solid phase technology of Dynabeads Oligo (dT)₂₅ eliminates the need for oligo (dT) cellulose columns. Pure cytoplasmic mRNA can be isolated from cell suspensions in 15 minutes, says Dynal. Recovery rates for mRNA of greater than 90 per cent are possible, while the fast reaction kinetics reduce the risks of physical and enzymatic degradation. mRNA isolated in this way can be used for northern blotting, cDNA synthesis, *in vitro* translation experiments, or as starting material for PCR reactions. Dynabeads Oligo (dT)₂₅ can be supplied in a \$190 (US) ready-to-use kit form, or as a \$500 (US) single reagent (5 × 1 ml containing 5 mg beads per ml, where about 2 μ g mRNA is isolated per mg beads). Optimal results are obtained when using Dynal's magnetic particle concentrator.

All you need for **RNA transcription and cDNA amplification** in a single tube is provided by the new GeneAmp RNA PCR kit from Perkin-Elmer (*Reader Service No. 107*). The kit can be used to generate a

cDNA product in excess of 2 Kb from all types of RNA (mRNA, tRNA, rRNA, total RNA and viruses) and to detect specific RNA species present at low copy number, says Perkin-Elmer. Reagents are supplied premixed and optimized for reverse transcription of RNA and subsequent amplification of the resulting cDNA products using the GeneAmp PCR process. Each kit includes the GeneAmp

RNA-PCR



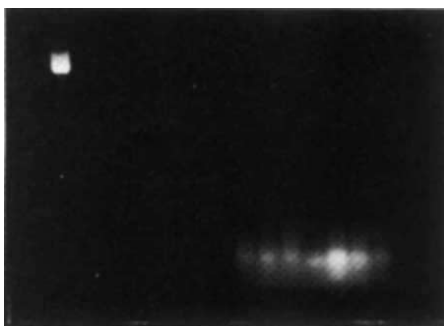
GeneAmp RNA PCR kit.

PCR core reagents, a positive control RNA template transcribed from the plasmid pAW 109, control primers that flank an Il-2 alpha sequence in the control RNA, random hexamers, reverse transcriptase, RNase inhibitor, dATP, dTTP, dCTP and dGTP. Sufficient positive control RNA is provided for 25 reverse transcription/amplification reactions starting with 10^3 copies per reaction.

Products for PCR

Clontech offers **PCR primers** for use in the GeneAmp PCR process for the amplification of DNA inserts contained in lambda vectors (*Reader Service No. 108*). Insert screening amplimers are available for lambda gt10, lambda gt11, lambda gt22, SWAJ-2, SWAJ-4, lambda BlueMid and lambda Zap vectors. Clontech's amplimers can be used to determine the size of the DNA inserts or to recover inserts that cannot be excised from the vector. Researchers pick a recombinant plaque, freeze/thaw it briefly, and then amplify the insert by PCR using the screening amplimers and the GeneAmp PCR reaction kit. The amplification product can be analysed on a gel, cloned into another vector, or sequenced. Each insert screening amplimer consists of a pair of primers and a detailed protocol.

The OptiTaQ kit from Bios makes light work of **determining the optimal buffer to use with specific primer:target DNA pairs** in a PCR experiment (*Reader Service No. 109*). Excessive or insufficient amounts of magnesium can interfere with the specificity and yield of PCR results, says Bios.



OptiTaQ kit for PCR — all you need to optimize buffer conditions.

The magnesium content required for a specific primer:target DNA pair will also vary according to the source of *Taq* polymerase and the method of preparation and/or storage of the DNA template. The kit includes 1 ml each of ten different $10\times$ buffers with varying concentrations of $MgCl_2$ (5 mM to 50 mM). A series of reactions using each of the OptiTaQ buffers are set up for each primer:target DNA pair. Normal thermocycling procedures are followed to produce PCR products. An aliquot from each series of reactions is run on an agarose gel and the buffer yielding the the greatest amount of specific product is determined.

Protein isolation/purification

Rainin Instruments has introduced a new **protein fractionator** — the Model RF3 — which is designed to provide gram-scale purification of proteins in 2–4 hours (*Reader Service No. 110*). Separation occurs by isoelectric focusing in free solution, without gels, solid supports or mesh separators, and operation is unaffected by isoelectric precipitation. With the RF3, the sample solution flows rapidly through a thin-channel electrophoretic cell; this set up provides a highly stable laminar flow that prevents convective mixing, says Rainin. Short residence times and an efficient heat exchange system maintain controlled operating conditions. A 30-tube fraction collector is supplied with the Model RF3.

The **ultrafast microprotein analyser** (UMA) from Michrom BioResources is a fully integrated microbore HPLC-based system designed for specialized applications (*Reader Service No. 111*). The system consists of a binary gradient microbore solvent delivery system, a 10-port automated sample injector, a thermally-controlled mobile phase column and flow-cell system, UV/visible detector with custom micro flow cell, and a PC/AT controller/data system. Two applications packages are available. The first, for the real-time monitoring of peptides and proteins produced either synthetically or by recombinant DNA techniques. The second, designed for the micropreparative purification and isolation of peptides and proteins at the 5–1,000-pmol level.

Michrom says that recoveries of greater than 90 per cent for peptides and proteins are possible at the 5 pmol level, with collection volumes of 10–25 μ l.

Cell culture

Mycoplasma Experience offers a complete **mycoplasma testing service** for the detection of mycoplasma contamination in cell cultures, sera, vaccines and biologicals (*Reader Service No. 112*). The company says its own brand of recovery media allows for the rapid growth of common cell culture contaminants, including strains that cannot be grown on conventional mycoplasma media. Mycoplasma Experience offers full technical support, confidential consultations and contract research and development, as well as a cost-effective voucher scheme for regular customers.

For high-volume culture work, New Brunswick Scientific has introduced the PourMatic carousel-type, self-stacking, **automatic agar dispenser** (*Reader Service No. 113*). PourMatic can pour and stack up to 320 petri dishes in 20 minutes, says NBS. The instrument can be set to dispense between 5 and 20 ml of agar in 1-ml increments and with an accuracy of ± 5 per cent, says NBS. A complete and compact plate preparation system can be put together by interfacing the PourMatic to a sterilizer. This set up is designed to both save time and effort in the laboratory and ensure that the agar plates are of a consistent quality with virtually no contamination.

Measuring 10.5 inches in diameter and 13.25 inches in height, the Ritter M-8 EasyClave self-contained **steam sterilizer** from Midmark is designed to be lightweight, portable and useful in laboratories where space is at a premium (*Reader Service No. 114*). No plumbing is required. To operate, the user must fill the sterilizer with distilled water, insert the instruments, close the lid and start. Sterilization cycle times take 25 minutes, says Midmark. Safety features include a self-

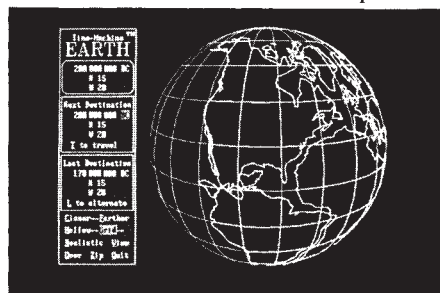


Steam clean with the M-8 EasyClave.

locking cover, pressure control valves and a low-water indicator system.

Software smarts

Time-Machine Earth is software from Sageware that is designed for **plate-tectonic modelling** on an IBM PC or compatible (*Reader Service No. 115*). Two versions are available to meet the differing needs of users: a personal edition that can be used as a teaching tool and a more sophisticated professional edition that, although it has many features in common with the personal edition, is aimed at a research level. Time-Machine Earth simulates the geological history of the planet and displays computer-generated satellite images of the globe showing the positions of the oceans and continents at a selected time period. According to Sageware, users can look at the earth up to 550



Time-Machine Earth software brings plate-tectonic modelling to PCs.

million years ago or 40 million years into the future with accuracy. A speculative look can be taken at the earth up to 2,000 million years in the past or future. Teachers can use the \$69.95 (US) personal version to demonstrate the fundamentals of continental drift. Using the 'blink' comparison, users can move between two eras with a keystroke. Alternatively, time periods can be superimposed. The \$245 (US) professional version allows the user to change the placement of continents in order to create maps based on alternate theories of continental movement. Each land mass can have its own independent pole and angle of rotation.

Watermaze, developed by Edinburgh-based Watermaze Software and available from Paul Fray, is **software for automatically tracking and analysing the path of a swimming rat** (*Reader Service No. 116*). The software, which runs on a 32-bit Acorn Archimedes computer, provides a simple means of monitoring the cognitive effects of various drugs and can be used as a tool for basic research involving spatial learning. Animals are monitored via a ceiling-mounted video camera coupled to an image analysing device and computer. An online interpolation routine smooths occasional tracking errors that can occur when the rat swims underwater. The path taken, together with the interpolated values, are displayed in real time and can be printed or stored on disk.

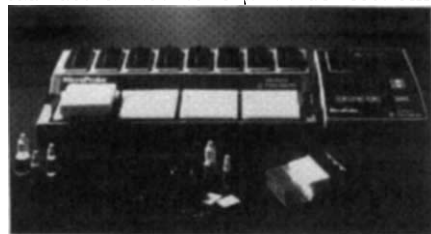
Mix it

The gentle two-dimensional action of the Robbins Scientific **mixer**, which is available from Nycomed, is designed to mix thoroughly solutions or blood-cell suspensions (*Reader Service No. 117*). As tubes are both rotated at 18 r.p.m. and rocked up and down, the mixer is particularly useful for keeping insoluble materials, such as separating beads, in suspension. Two models accommodating twelve 10-17-mm tubes or eight 50-ml tubes are available. The mixer heads are interchangeable so that both sizes of tube can be used.

Jencons Scientific's **four-plate orbital shaker**, which is designed for mixing without cell disruption, is a handy benchtop aid (*Reader Service No. 118*). The microplate mixer, costing less than £350 (UK), has a shaking speed that can be set to between 0 and 1,800 r.p.m. An optional extra is the 1-30-minute timer.

Benchtop aids

The MicroProbe **manual slide stainer** from Fisher Scientific is designed to provide greater uniformity, reproducibility and speed to chemical staining, immunohistochemical and *in situ* hybridization procedures (*Reader Service No. 119*). The \$2,595 (US) MicroProbe system, with its 10 × 26-inch footprint, has three major components: a slide holder, staining module and incubator. The slide holder has slots for up to ten pairs of special slides and so 20 specimens can be processed simultaneously. The gap between each slide pair, which fills by capillary action, can accommodate 5-μ tissue sections.



MicroProbe: Fisher Scientific's all-in-one slide stainer and incubator.

The instrument's staining module has eight stations for bulk-type dewaxing and staining reagents and four stations for use with more expensive immunoreagents, DNA probes or blotting pads. User-programmable temperature control from 30 to 110°C, with an accuracy of ± 0.5°C, is possible with the system's microprocessor-controlled incubator.

With safety precautions in mind, Owl Scientific has a **radiation safety rack** for storing tubes containing beta-emitting isotopes (*Reader Service No. 120*). The \$49 (US) rack box comes with an 80-slot microtube rack, but can also accommodate scissors, markers and other small laboratory utensils. It is constructed of

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clear, heavy-duty 0.375-inch thick acrylic and measures 9.75×3.375×3.25 inches. The box is equipped with a tight-fitting lid and four 'non-skid' rubber feet. Custom box designs are available on request.

Mitsubishi Kasei has launched the compact Model GT-06 **automatic titrator**, which can be configured to perform all types of titrations — acid-base, redox, argentometric, chelatometric, non-

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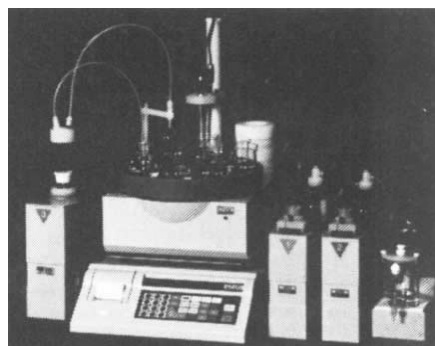
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Model GT-06 modular titration system from Mitsubishi Kasei.

aqueous, or photometric (*Reader Service No. 121*). The basic system comes with a standard built-in printer and RS232 interface for linking it up to a balance, and with an optional bi-directional computer communication. For ease and speed of operation, up to eight titration files can be stored in the systems's memory. When the Model GT-06 is linked to the Model GT-LD photometric detector, users can perform photometric and chelatometric titrations, and titrations using an indicator. Another option is the Karl Fisher titration unit.

Information sources

Scientists involved in connective tissue and blood anticoagulant research may be interested in the **1990 biochemical catalogue of leech-derived products** from Biopharm (*Reader Service No. 122*). The catalogue features a host of enzyme and protease inhibitors, including hirudin (a potent inhibitor of thrombin), leech collagenase (a collagen-specific enzyme), leech hyaluronidase (an endo-β-glucuronidase) and hementin (a fibrinogenolytic enzyme).

Keep up to date with the latest **catalogue updates** from the American Type Culture Collection (ATCC) (*Reader Service No. 123*). The 38-page update to ATCC's 1989 bacteria and bacteriophages catalogue includes information on 450 new strains, recommended procedures for reviving preserved cultures, methods for storing cultures, and media formulations for use in subculturing each strain. In addition, an index of about 650 strain designations in use by other laboratories and culture collections should provide a

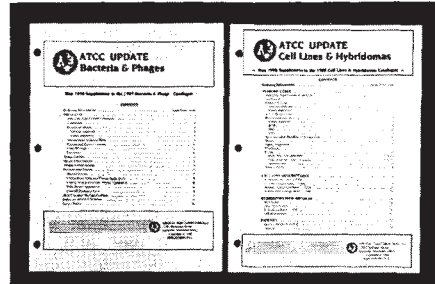
useful aid in the location of a particular strain. The 53-page cell line and hybridoma update to ATCC's 1988 catalogue lists an additional 80 cell lines and 170 hybridomas. An index to the antigenic determinants that react with the mAbs produced by the hybridomas and a species/tissue index to the cell lines are included. Copies of both updates are free on request and are available via ATCC's online computer service.

Have you ever had cause to look for an organism with very precise properties? Yes, well the Institute of Biotechnology's **24-hour online information service** — the Microbial Strain Data Network (MSDN) — for yeasts, algae, bacteria, cell lines, hybridomas, recombinant clones and libraries may be the answer (*Reader Service No. 124*). The MSDN combines a directory to information services with related databases — catalogues, primary laboratory data and other information centres. As the information service is linked up to an electronic mail service, users can place orders without exiting the system. The service is available online or through the MSDN Secretariat and costs £25 (UK) for registration and documentation: subsequent charges are made for online use. A typical five-minute search during prime time would cost about £2.15 (UK), says IOB. In addition, MSDN and DATASTAR have entered into an agreement to provide an electronic gateway between the two services. DATASTAR is offered at a discounted rate to MSDN users. This service provides access to databases in biomedicine, chemistry and biotechnology, including Medline, Scisearch, Biosis, Chemical Abstracts, Current Biotechnology Abstracts, Computer Database, and Excerpta Medica.

An extensive range of laboratory consumables and equipment for **sample handling** tasks are listed in the new Pierce and Warriner catalogue (*Reader Service No. 125*). A vast array of vials, ampoules, flasks, syringes, closures, seals, discs and septa are featured in the free catalogue. Also included are stirrers, heaters and evaporators, as well as equipment for filtration and dialysis.

Sigma has a line of **gold conjugates** which in analytical procedures provide an alternative to RIAs and ELISAs (*Reader Service No. 126*). Greater than 50 products are offered in three complementary/interactive systems: avidin/biotin; lectin/glycoprotein; primary and secondary antibodies/Protein A and G.

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.



ATCC's catalogue updates — now free on request or online.

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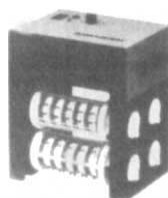
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